QP 84 .A1 G88

Official Organ of the Society for the Study of Development and Growth

GROWTH

A JOURNAL FOR STUDIES OF DEVELOPMENT AND INCREASE

UNIVERSITY OF MICHIGAN
APR 8 1958
SCIENCE LIBRARY

VOLUME XXI, NUMBER 4
PAPERS 526-531

DECEMBER, 1957 $6.00 A YEAR

Second-Class mail privileges authorized at Worcester, Mass.

GROWTH

A journal for the coordination of studies of increase and development as general properties of nature. Published by contributors and subscribers on a cooperative, non-profit, non-salary basis. The field of investigation is primary and work will be published in which the approach is through mathematics, physics, chemistry, genetics, embryology, philosophy, and anthropology; just as will studies of development in its several expressions, and of mass increase and the part played therein by nutrition, vitamins, and hormones.

It is published four times a year during March, June, September and December at $6.00 per volume (outside U. S. and Canada, $6.50). Single numbers $2.00. There will be one volume each year.

All manuscripts and subscriptions should be sent to: The Editors of Growth, Lankenau Hospital Research Institute, Fox Chase, Philadelphia 11, Pa.

THE EDITORS OF GROWTH

Managing Editor

STANLEY P. REIMANN, M.D.

Lankenau Hospital Research Institute and Institute for Cancer Research, Fox Chase, Philadelphia, Pa.

Editor-in-Chief

IRENE COREY DILLER, PH.D.

Institute for Cancer Research, Fox Chase, Philadelphia, Pa.

John T. Bonner, Princeton University
Donald P. Costello, Univ. of N. Carolina
S. A. Courtis, University of Michigan
Sears Crowell, Indiana University
Harry G. Day, Indiana University
Earl L. Green, Ohio State University
Wm. W. Greulich, Stanford University
Leigh Hoadley, Harvard University
Warren H. Lewis, The Wistar Institute
Leo Loeb, Washington University
Willard C. Olson, University of Michigan
Earle L. Reynolds, Atomic Bomb Casualty Comm'n
E. W. Shrigley, Indiana University Medical Center
E. W. Sinnott, Yale University
Philip R. White, Roscoe B. Jackson Memorial Laboratory

Corresponding Editors

B. Boysen Jensen, Univ. of Copenhagen
Ivar Broman, Tornblad Institutet
Luigi Castaldi, Università di Cagliari
A. Dalcq, Université de Bruxelles
E. Fauré-Fremiet, Collège de France
Alexander Haddow, Chester Beatty Research Institute
Julian S. Huxley, Zoölogical Society
Jacques Monod, Institut Pasteur
Joseph Needham, Caius College
Yo. K. Okada, Kyoto Imperial University
G. Teissier, Faculté des Sciences
A. H. Roffo, Universidad de Buenos Aires

Growth, 1957, **21**, 209-220.

RELATIONS OF ORGANIC PHOSPHORUS DISTRIBUTIONS IN TESTES TO P^{32} ADMINISTRATION, TESTIS GROWTH, AND HORMONAL EFFECTS[1]

PAUL L. RISLEY AND A. L. SODERWALL

Biology Department, University of Oregon, Eugene, Oregon

(Received August 9, 1957)

INTRODUCTION

SEVERAL authors have observed alterations in lipid phosphorus following irradiation, and other experimental conditions. McEnery and Nelson (1953b) mention this as a possible effect following x-irradiation of rat testes, and also after cryptorchidism. Milch and co-workers (1954) report elevated blood lipid phosphorus values after partial body x-irradiation. Riedel and Rossiter (1954) obtained decreased lipid P values for adrenal glands following hypophysectomy [also observed by McEnery and Nelson (1953b) in testes]; lowered P^{32} lipid incorporation was also observed, with restoration of normal levels after ACTH therapy. Borell, Diczfalusy, and Westman (1952) studied P^{31} and P^{32} distributions in normal and hypophysectomized rat testes.

The purpose of the present paper is to present some comparative results obtained for organic phosphorus (P) and P^{32} distributions in hamster testes under several conditions. Comparisons of the distributions in acid-soluble (ASP), lipid (LP), and residual protein (RPP) phosphorus fractions in testes of several groups of animals were made on an individual whole testis basis. It was assumed that differences in the relative quantities of the phosphorus-containing compounds might prove useful in evaluating altered metabolic states in the testicular tissues under the conditions being studied. This assumption appears consistent with recent conclusions of Riedel and Rossiter (1954) for the adrenal glands of rats, and the well-known testis-adrenal relations provide an interesting point of comparison in regard to the general problem of phosphorus metabolism in these organs.

[1] Research aided by grants from the United States Atomic Energy Commission. Assistance of those individuals aiding with the work during its progress is also acknowledged.

Materials and Methods

A limited number of male hamsters furnished 24 testis samples for complete analyses. The animals were separated into four groups as follows: I—5 normal adults (average body weight:—87.7 g.); II—4 hemicastrates (60 days; average body weight:—81.2g.); III—27-32 day old normal immature (average body weight:—41.2 g.); and IV—27-32 day old young treated with testosterone propionate (average body weight:—42.0 g.). In the last group, each animal received ten injections of 0.25 mg. testosterone propionate (Schering-OretonF) over a twenty-day period from seven to twenty-seven days of age, when P^{32} was injected intraperitoneally.

In all cases, forty-eight hours elapsed from the time of P^{32} injection to the time of autopsy. P^{32} values obtained are adjusted for background, decay, and to an equivalent 1.22 μC per gram body weight initial dose. Mean values and standard deviations for single whole testis samples are given for each of the four animal groups, and for acid-soluble, lipid, and residual protein phosphorus fractions in the tabular summary. The data are expressed also in specific activities ($SA \times 10^4$), or the ratios of P^{32} in disintegrations per minute to the P values in mg. Blood samples were taken for each animal and blood specific activities (RSA) were calculated for each testis fraction in relation to the blood phosphorus fraction for each group of animals. Likewise, the percentage distributions of both P and P^{32} in the several fractions were determined. For purposes of comparison, the several bases of measurement prove useful in the considerations which follow.

One testis from each animal was used for P and P^{32} determinations, and the same samples were used for both. P determinations were made following fractionation procedures recommended by McCarter and Stelges (1948), Schmidt and Thannheuser (1945), and Borell (1951). Trichloracetic acid was used for tissue oxidations in the fractionation procedures prior to the formation of phosphomolybdic acid for P determinations. Values are expressed in mg. P per single testis sample as compared with a measured 0.03 mg. P standard and a blank at the times of colorimetric measurement with the Klett-Summerson apparatus. P^{32} was measured by means of a liquid dipping Geiger counting tube of Tracerlab "TGC-5" type with a Tracerlab "64" Scaler. Results are expressed as disintegrations per minute, after corrections for dilution and decay. The other testis of each animal was used for histological study in order to check the normality

or pathology of the tissues. Routine methods of histological preparation were employed, using Bouin's fluid as a fixative and Harris' Haematoxylin and Eosin as stains. Normal expected tissue conditions were encountered in all cases, and β- radiation effects were not recognized after a 48-hour period of exposure.

Observations

The data obtained for the several groups of animals tested are summarized in Tables 1, 2, 3, and 4. No significant differences appeared between the normal and hemicastrate adults (Groups I and II) in the acid-soluble P mean absolute values. A slight variation is observed, however, between these groups in a higher ASP percentage of the total P in the hemicastrate testis, and a somewhat lower % ASP^{32} than in the normal adult testis. The absolute mean values of ASP are lower for the normal young testis (III) and the testosterone treated testis (IV), but those of ASP^{32} were comparable to the normal adults. Testosterone-treated testes gave lowest values for ASP^{32}, but the specific activity for this group proved to be closer to that observed for the normal adults, suggesting a possible effect of the hormone treatment on the P and P^{32} balance in this fraction (see Borell, Diczfalusy, and Westman, 1952, for effects of chorionic gonadotrophin treatment after hypophysectomy).

In the lipid P absolute mean values, the young and hormone-treated young were similarly lower than those of the adult groups. Differences appeared in relation to size and age only. However, the LP^{32} values were distinctly higher in groups III and IV. High specific activity values clearly illustrate the differences between the young and adult testes in the lipid phosphorus fraction. A greater % LP^{32} appeared in the hormone-treated group, suggesting a possible effect of the male sex hormone, but more data are needed for confirmation of this point.

The residual protein phosphorus (RPP and RPP^{32}) fractions are reversed in order of magnitude with the normal adult testis high in RPP, but lowest in RPP^{32}. Again the difference appears to be one principally related to ages of the groups and testis size. As in the lipid fraction, specific activities are highest in groups III and IV, and lowest in groups I and II. The hormone-treated young of group IV gave lower absolute mean values in both P and P^{32}, but a trend toward the values observed for the adult groups was indicated also in the percentage protein phosphorus fraction, and the specific activity.

The data obtained clearly indicate that distinctly higher specific activities characterize the young testis as compared with the adult testis, both with respect to total phosphorus and the several fractions.

TABLE 1
Acid-soluble Phosphorus in Hamster Testes. Values Are Expressed as the Absolute Mean Values Per Testis with Standard Deviations for Each Group (as Described in Text).

Animal Group	Mg. P	$P^{32}c/m$	$SAx10^4$	%P	$\%P^{32}$
I.	0.1822±0.056	3390±1788	1.79±0.5	38.4	66.39
II.	0.1871±0.056	3146±1162	1.70±0.37	41.8	59.82
III.	0.1266±0.025	3375±1131	2.67±0.66	35.8	53.20
IV.	0.1351±0.051	2780±1068	2.10±0.41	39.4	52.16

TABLE 2
Lipid Phosphorus in Hamster Testes. Values Are Expressed as the Absolute Mean Value Per Testis with Standard Deviations for Each Group.

Animal Group	Mg. P	$P^{32}c/m$	$SAx10^4$	%P	$\%P^{32}$
I.	0.1279±0.052	1048±458	0.82±0.41	26.9	20.53
II.	0.1222±0.043	1353±556	1.10±0.16	27.3	25.72
III.	0.0958±0.036	1687±615	1.81±0.57	27.0	26.60
IV.	0.0904±0.030	1624±408	1.98±0.82	26.3	30.37

TABLE 3
Residual Protein Phosphorus in Hamster Testes. Values Are Expressed as the Absolute Mean Value Per Testis with Standard Deviations for Each Group.

Animal Group	Mg. P	$P^{32}c/m$	$SAx10^4$	%P	$\%P^{32}$
I.	0.1640±0.055	666±331	0.40±0.13	34.7	13.06
II.	0.1386±0.062	765±321	0.60±0.28	30.9	14.56
III.	0.1312±0.019	1282±468	0.97±0.28	37.1	20.20
IV.	0.1172±0.038	935±236	0.88±0.28	34.2	17.46

TABLE 4
Total Organic Phosphorus in Hamster Testes. Values Are Expressed as the Absolute Mean Value Per Testis with Standard Deviations for Each Group.

Animal Group	Mg. P	$P^{32}c/m$	$SAx10^4$	%P	$\%P^{32}$
I.	0.4741±0.101	5105±2439	1.06±0.38	100.0	100.0
II.	0.4479±0.150	5260±2008	1.19±0.29	100.0	100.0
III.	0.3536±0.068	6345±2020	1.80±0.46	100.0	100.0
IV.	0.3428±0.091	5349±1422	1.63±0.45	99.9	100.0

Differences between the normal adults (I) and the hemicastrates (II) were not pronounced enough to be regarded as significant. Similarly, differences between groups III and IV were not sufficiently great to be significant, and it appears that the hormone treatment was not adequate to differentiate the groups sharply and statistically. Certain

trends in the data, however, suggest that alterations of the percentage values of the several fractions may be more important indicators of the physiological conditions of the testes than are the absolute mean values that exhibit considerable variation within the group, and frequently overlap between the groups. Differences were not as great as might have been expected in the groups, perhaps because of insufficient initial differentiation in the establishment of the experimental groups. For example, the 30-32 day old hamster is just completing the initial spermatogenetic cycle; spermatozoa are present and entering the epididymis for the first time. Histologically, there would be little difference in the cellular state of the young and adult testes, but size and hormonal state do differ.

Discussion

McEnery and Nelson (1953 a, b) analyzed the rat testis for P distributions in the several fractions, including the desoxyribo- and ribonucleic acid fractions of the residual proteins under normal and experimental conditions and during the growth period. An examination of their data with reference to their conclusion that lipid P concentrations remain constant under various conditions, as compared with our own for the hamster testis after administration of P^{32}, indicates that percentage lipid phosphorus values increase as a result of irradiation effects. McEnery and Nelson (1953b) cautiously suggest that the lipid phosphorus of the testis may be increased directly as a result of x-irradiation and under conditions of cryptorchidism on the basis of high lipid P values after these treatments as compared to hypophysectomy and estrogen-treatments, where lower than normal values were found.

In order to compare the data of several authors, Table 5 gives the results of available analyses of organic phosphorus fractions of rat and hamster testes in mg. P per testis, and also of P^{32}, after conversion to percentage values of the several fractions in relation to the total phosphorus accounted for. It will be noted that in all cases where P^{32} was injected, the % LP was similarly high, as compared with the McEnery and Nelson (1953a) values for normal adult testes (20.7% and 18.8% LP^{31} for 60- and 180-day-old rats, respectively), and with our 20.5% for LP^{32} content in adult hamster testes. Only in 30-day post-x-irradiated and 200-day cryptorchid rats did the McEnery and Nelson (1953b) values for % LP approach those obtained by others

TABLE 5

Summary of Data by Several Investigators on Testis Phosphorus. Recalculated to % Distribution in Fractions Reported.

Authors and Experiments	ASP		LP		RPP		Remarks
	Mg.	%	Mg.	%	Mg.	%	
McCarter and Stelges (1948)	0.740	36.8	0.580	28.8	0.690	39.3	Rat (P^{31}/gr. wet wt.)
McEnery and Nelson (1953)							
A. 10 days	0.030	45.8	0.007	10.5	0.029	43.6	Rat (Mg./testis)
B. 20 days	0.132	41.9	0.039	12.3	0.144	45.7	
C. 30 days	0.272	40.0	0.096	14.1	0.311	45.7	
D. 40 days	0.637	38.7	0.292	17.7	0.716	43.5	
E. 60 days	0.784	35.2	0.432	20.7	0.919	43.6	
F. 180 days	1.026	37.7	0.512	18.8	1.183	43.4	RPP^{31} given as TNAP (Nucleic acid fractions)
Hypophysectomized	0.254	35.9	0.108	15.2	0.335	48.5	
Estrogen treated	0.365	40.1	0.127	14.0	0.417	45.5	
X-irradiated	0.300	36.4	0.208	25.2	0.317	38.4	
Cryptorchid (80 day)	0.282	40.0	0.138	19.5	0.285	40.4	
Cryptorchid (200 day)	0.261	32.3	0.224	27.2	0.339	41.1	
Borell, Diczfalusy, Westman (1952)							Rat (Mg./testis)
Hypophysectomized immature	0.076	34.9	0.052	27.4	0.061	32.3	7% loss in ASP fractions
Chorionic gonadotrophin	0.091	38.4	0.067	28.4	0.078	33.0	21% loss in ASP fractions
Risley, Soderwall							Hamster (Mg./testis)
I. 75-180 day adults	0.182	38.47	0.127	26.97	0.164	34.6	Calculated absolute values
II. Hemicastrates	0.187	41.77	0.122	27.26	0.138	30.9	
III. 27-32 day young	0.126	35.80	0.095	27.09	0.131	37.1	
IV. Testosterone treated young	0.135	39.41	0.090	26.37	0.117	34.2	

TABLE 5 (*continued*)
Summary of Data by Several Investigators on Testis Phosphorus. Recalculated to % Distribution in Fractions Reported.

Authors	ASP^{32}		LP^{32}		RPP^{32}		Remarks
	Mg.	%	Mg.	%	Mg.	%	
McCarter and Stelges (1948)	0.052	74.28	0.013	17.14	0.006	8.58	%P^{32} dose/gr. tissue
Risley and Soderwall							
I. 75-180 d. adult	3390	66.39	1048	20.53	666	13.08	P^{32} distintegrations
II. Hemicastrates	3146	59.81	1353	25.72	765	14.47	/ minute / testis.
III. 27-32 d. young	2592	53.19	1296	26.59	984	20.22	
IV. Androgen treated	1886	52.15	1099	30.36	633	17.49	
Borell, et al. (1952)				S.A.		S.A.	
Hypophysectomized young	—	—	—	2.0	—	2.4	Data given only as specific
Chorionic gonadotrophin	—	—	—	6.2	—	3.5	activity

who used P^{32} (McCarter and Stelges, 1948) (Borell, Diczfalusy, and Westman, 1953) and ourselves. It therefore seems conclusive that the administration of P^{32} by injection results in an elevated percentage of lipid P, and that this effect is evidently attributable to β-radiation.

The effect of P^{32} β-radiation in producing an increasing % lipid P (about 6-8%) must be immediate and continuous. In the experiments of Borell et al. (1952), only three hours elapsed between P^{32} administration and autopsy. McCarter and Stelges (1948) used a 24-hour period, and our data were obtained after 48 hours. It is evident that the effect is not a transitory one, but is noted at least over the two-day period for which data is available, and in x-irradiation determinations, after a 30-day period. Further investigations to clarify the nature of this radiation effect on the testis would seem desirable, and more conclusive experiments are needed.

The observations of Milch and co-workers (1954) that following body x-irradiation there is an elevated blood lipid P and a lowered blood cholesterol in the rabbit is worth noting in relation to the changes observed in the whole testis. A question arises as to whether the observed elevation of the testis % LP may be due to changes in blood lipid P or in the testicular tissues themselves. Since much higher radiation dosages were used in the x-ray experiments on the blood lipid P than in those involving P^{32} treatments, it seems doubtful that increased testis % lipid P could be accounted for by blood remaining in the testis at the times of analysis.

Further study of the data of McEnery and Nelson (1953a, b) on the relative quantities of phosphorus in the several chemical fractions of the testis under normal and experimental conditions enables a summary of their findings in relation to the growth of the testis in Figure 1. Taking their absolute mean values for testis weight and the several phosphorus fractions, the increase in each has been calculated in terms of the number of times the values of each for the 10-day old testis, which, therefore, serves as a base unit of one. Several interesting relations appear when a comparison is made in this simple way. First, there is a direct parallelism between testis weight and lipid P increase from the 10-day to 180-day testis, except for a slight decrement in the latter. Second, the markedly high LP levels of the normal adult testis are evidently significant in relation to testicular function, as compared to the ASP. Third, once the adult phase is attained, the relative phosphorus quantities ap-

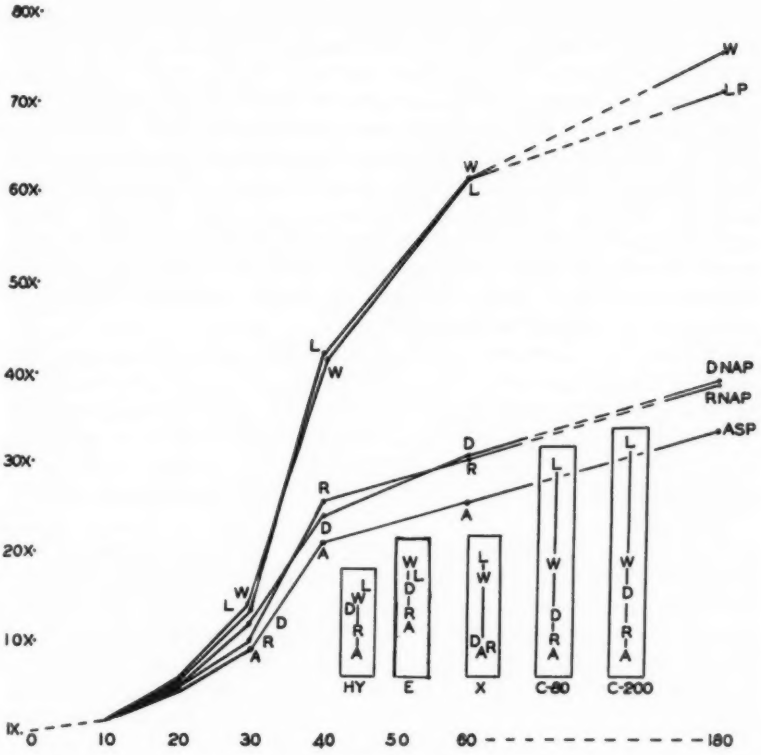


FIGURE 1

Graph showing distributions of the organic phosphorus fractions during normal growth of the rat testis and after experimental treatments. (After McEnery and Nelson, 1953a, b). Ordinate values are calculated relative to the 10-day old rat testis as IX; age is indicated in days on the abscissae.

Legend:

W	— testis weight
L	— lipid phosphorus
A	— acid-soluble phosphorus
R	— ribonucleic acid phosphorus
D	— desoxyribonucleic acid phosphorus
H	— after hypophysectomy
E	— estrogen-treated
X	— 30 days post-X-irradiated
C80	— Cryptorchid (80 days)
C200	— Cryptorchid (200 days)

parently become stabilized in the several fractions. Fourth, an abrupt rise in growth begins at the thirtieth day and continues to the fortieth day in all fractions, but it is noted that the LP rises most sharply and continues to increase rapidly between the fortieth and sixtieth days, when the LP becomes stabilized and parallels the ASP and nucleic acid P fractions in further growth. The changes noted are coincident evidently with the completion of initial phases of spermatogenesis near the fortieth day and the attainment of adult hormone output near the sixtieth day. The continuing lipid phosphorus increase between 40 and 60 days of age possibly could be associated with the attainment of adult levels of lipid and steroid hormonal metabolism necessary for a complete breeding state.

Leblond and Clermont (1953) describe the germ cell cycle of the rat and hamster as consisting of five spermatogonial divisions that produce 24 spermatocytes and one stem cell for the next generation of spermatocytes. It is, therefore, of considerable interest to note that the nucleic acid P increases are almost exactly coincident at 25 $\times$ the 10-day figure at forty days of age, when the first spermatogenic cycle is in progress.

In the lower right-hand area of Figure 1, the relative quantities of the several fractions of phosphorus compounds of the rat testis under experimental conditions are indicated from the data reported by McEnery and Nelson (1953b). It is interesting to observe that testis weight (W), lipid P, acid-soluble P (A), desoxyribose nucleic acid P (D), and ribonucleic acid P (R) all approach the levels of the thirty-day testis, except for slight variations. Hypophysectomized adult rats conform closely to the thirty-day old testis, but estrogen treatments give slightly higher comparative values. After x-irradiation and cryptorchidism lipid P levels remain highest even in the eighty-day cryptorchid testis. After x-irradiation nucleic acid P levels are lowest, reflecting the well-known sensitivity of germinal cells to this treatment, and a probable nucleic acid-spermatogenetic cycle relationship, as discussed in detail by McEnery and Nelson.

Although McEnery and Nelson (1953a) report no alterations in lipid P concentrations in their ten- to 180-day series of normal rat testes, an increase in absolute LP quantities coincided with growth and age changes. Reference to Table 5 demonstrates that the data of McEnery and Nelson also show a progressive increase in % LP with development and growth of the testis. This fact was not mentioned by

them and it is noteworthy that a nearly comparable decrease occurred in the % acid-soluble P with growth and development. Nucleic acid % P values decreased slightly. The greatest increase in % LP, however, appears in their developmental series between the thirtieth and fortieth days of age (when active spermatogenesis begins) and continues through the sixtieth day when male sex hormone production of the adult age is nearly attained. Unfortunately, it remains difficult to segregate spermatogenesis and hormone production in time or by experimental means in order to establish more precise correlations. The alterations noted in the relative amounts of lipid phosphorus in testis growth must be interpreted, therefore, as correlated with (1) growth in size and increase in cell numbers, (2) steroid metabolism in relation to androgen synthesis, and (3) as yet uncertain reactions relating to metabolic adjustments probably associated with hypophyseal functions. The latter conclusion seems justifiable on the basis of the observed percentage changes in the relative distributions of the several fractions after beta- and x-irradiation, and the correlation of stable organic phosphorus percentages with the attainment of sexual maturity.

Beta-radiation effects following injections of P^{32} are apparently similar to effects of partial body x-irradiation, in that % LP values of the testis are increased. It is also interesting to note that this response is rapid in occurrence after P^{32} injections, and that a similar effect is observed in both irradiated and cryptorchid testes. The increased percentage lipid P seems directly related to the accompanying metabolic changes in the testis and it may be suggested that this fraction could provide a useful index for measurement of testis growth responses in experimental studies. Riedel and Rossiter (1954) find a similar effect in adrenal glands after hypophysectomy, and arrive at a similar generalization regarding lipid phosphorus changes and metabolic alterations.

Summary

The available published data on lipid phosphorus of the testis after treatments with P^{32} indicate that the percentage lipid P is increased about 6-8% over normal, and that the percentage acid-soluble P is reduced. A comparison with the results of McEnery and Nelson (1953a, b) after x-irradiation and cryptorchidism suggests that increased percentage lipid P is a radiation effect following P^{32} administration by injection. No differences were noted in the testis lipid P

percentages in young, adult, or testosterone-treated young hamsters following P^{32} injections. A marked increase in % LP occurs during normal testis growth between the 30th and 60th day, and is coincident with normal testis weight increase in the rat.

Testosterone-treated young hamsters gave lower total P^{32} values, notably in the ASP^{32} fraction. In comparison with the untreated young testes, lower P^{32} values and specific activities were observed in all fractions.

The observed values suggest that the testosterone-treated animals more closely resembled the adult groups than the normal young of the same age, which exhibited the highest specific activities of the groups tested.

References

Borell, U., Diczfalusy, E., & Westman, A. 1952. Effect of chorionic gonadotrophin on turnover of phosphorus in testicles of hypophysectomized rats. *Acta Endocrin.*, **9**, 59-68.

———. 1951. Influence of ovarian hormones on acid-soluble phosphate of endometrium. *Acta Endocrin.*, **7**, 17-130.

Leblond, C. P., & Clermont, Y. 1953. Renewal of spermatogonia in the rat. *Amer. J. Anat.*, **93**, 475-498.

McCarter, J. A., & Stelges, E. J. 1948. Methods for the determination of the distribution of radioactive phosphorus among the phosphorus-containing constituents of tissues. *Canad. J. Research, E.*, **26**, 333-346.

McEnery, W. B., & Nelson, W. O. 1953a. Organic phosphorus compounds in the testis of the rat at various ages. *Endocrin.*, **52**, 93-103.

———. 1953b. Organic phosphorus compounds in the testis of the rat under experimental conditions which impair spermatogenesis. *Endocrin.*, **52**, 104-114.

Milch, L. J., Yarnell, R. A., Stinson, J. V., & the Cardiovascular Research Group. 1954. Relationship between blood lipids and radiation injury in rabbits. *Science, N.S.*, **120**, 713-714.

Riedel, B. E., & Rossiter, R. J. 1954. Phosphorus metabolism of the adrenal gland. The mechanism of the change after hypophysectomy. *Canad. J. Biochem. & Physiol.*, **32**, 261-270.

Schmidt, G., & Thannheuser, S. J. 1945. A method for the determination of desoxyribonucleic acid, ribonucleic acid and phosphoprotein in animal tissues. *J. Biol. Chem.*, **161**, 83-89.

Growth, 1957, **21**, 221-237.

ASCITES TUMOR DEVELOPMENT. III. ANALYSIS OF AN ANTISERUM PREPARED AGAINST THE SOLUBLE PHASE OF THE CELL[1]

EDWARD C. HORN*

Department of Zoology, Duke University, Durham, North Carolina and Biology Division, Oak Ridge National Laboratory,[2] Oak Ridge, Tennessee

(Received September 2, 1957)

INTRODUCTION

THE cytotoxic effects on living tissues of antisera prepared in mammalian hosts by inoculating homogenates of these tissues have been described numerous times in the literature. In a relatively few instances (Dulaney et al., 1949; Malmgren et al., 1951; Colter et al., 1956; Horn, 1956), a specific particulate component from essentially one cell type or whole cells of one type were used as the antigen, and the cytotoxicity of the antiserum elicited toward the specific cell strain donating the antigen was demonstrated. Studies of this type naturally preclude the identification of the specific antigen or antigens involved because of their inherent complexity.

A cellular preparation more amenable to quantitative and perhaps even qualitative antigenic analysis is known as the "saline-soluble fraction" of a cell or tissue; i.e., the supernatant fraction of a brei formed in physiological saline by any of a number of disrupting tools and subjected to a wide range of centrifugal forces. Some work with an antigen prepared in this manner from cancer cells (e.g., Colter et al., 1956) has been reported, but a more abundant and in most instances a more fundamental type of research on cytotoxicity with an antigen of this type has been reported in the embryological literature. Antisera to variously prepared saline-soluble fractions of embryos have been used to treat developing embryos of different ages and have been shown to produce a variety of cytotoxic effects usually

* Consultant, under Subcontract No. 796.

[1] This work was supported in part by research grant RG-4203 from the National Institutes of Health, Public Health Service.

[2] Operated by Union Carbide Nuclear Company for the U. S. Atomic Energy Commission.

resulting in, or at least coincident with, the death of the embryo at a particular stage (Burke et al., 1944; Parkes, 1946; Ebert, 1950; Flickinger and Nace, 1952; Clayton, 1953). The physicochemical analysis of these antigenic mixtures could ultimately lead to the identification of the specific antigen or antigens that elicit a cytotoxic antiserum.

This study was based on the same rationale as those investigations already mentioned; namely, that a study of the macromolecules of the cytoplasm is the most fruitful route to the identification of a cellular antigen or antigens that stimulate production of cytotoxic antisera. The "soluble phase" of Anderson (1956) was chosen here as the antigenic mixture because it was assumed to contain fewer antigens than whole breis, having lost all particulates by sedimentation. In addition, the use of a single cell type, the Ehrlich ascites tumor cell, for preparation of antigen[3] and testing seemed to have certain obvious advantages over the saline-soluble breis prepared from solid tumors or minced embryos with their heterogeneity of cell types and presumed additional subtle macromolecular differences.

Materials and Methods

Mice.—All mice used in these experiments were male and female inbred C_3H strain supplied by Cumberland View Farms, Clinton, Tennessee.

Preparation of antigen.[3]—Ehrlich ascites tumor cells to be used for the preparation of antigen were collected in dilute Versene[4] from a few mice, providing 10-15 ml of a milky ascitic fluid. The pooled cells were diluted with saline and strained through silk bolting cloth to remove small clots, after which they were washed several times by successive centrifugations and resuspensions in fresh 0.15M NaCl. After the final wash, the remaining erythrocytes formed a thin upper layer easily aspirated with a pipette. The packed tumor cells were then suspended in several volumes of 0.25 M sucrose and with successive centrifuging and suspension were washed several times in the sucrose solution. The final packed volume of cells (2000 rpm, average relative centrifugal force of 800) was resuspended in sucrose in a 1:5

[3] "Antigen" used generically and in the singular to designate the inoculant in these experiments with the knowledge (and ignorance) of its multiplicity and heterogeneity.

[4] A stock solution of 0.3M disodium versonate in 0.15 M NaCl was used to dilute the collected ascites fluid in a ratio of 0.01 ml of stock per 1 ml of ascites fluid.

dilution and blended in 10 ml samples in a microhomogenizer,[5] with the blending cup packed in ice during the 4- to 5-minutes' homogenization. The product was then centrifuged for 1 hour at 40,000 rpm, this providing an average gravitational field of 105,400 times.[6] This centrifugal treatment has been shown to remove all nitrogen-containing particles from such a preparation within 15 minutes (Anderson, 1956). A crust of fatty material was discarded from the upper portion, and the main volume of clear fluid was then removed carefully from a small pellet. Micro-Kjeldahl nitrogen determinations were immediately accomplished for each such supernatant preparation. The supernatant, without further treatment or storage, was injected intravenously into rabbits. All steps in the preparation of the antigen were carried out at 0-4° C.

Immunization schedule.—Each rabbit was injected intravenously with antigen on alternate days for two weeks, receiving a total of approximately 60 mg protein. Freshly prepared antigen was used for each injection. Since the protein content per unit sample varied considerably from preparation to preparation, the volume of each supernatant antigen injected was calculated to provide the individual rabbit with essentially the same quantity of protein per injection. After two weeks injections were stopped for a week. On the twenty-first day of the schedule a small blood sample was taken from each animal, the serum collected, and a complement-fixation titer obtained against a known concentration of freshly prepared antigen. This primary bleeding always gave a low titer. On the thirty-fifth day the animals were challenged with a single intravenous injection of approximately 60 mg of protein. A week later the animals were bled and again tested by complement fixation (CF). Since the titer had invariably improved considerably, the animals were bled for a reasonably large volume; the serum was collected, merthiolate added, and stored under refrigeration. In these experiments we used only this antisupernatant serum (AS) obtained after a secondary injection. The rabbits were challenged several weeks later with another dose of approximately the same protein content and, after an additional week, were bled to death. A test of the CF activity of this serum showed it to have a titer not exceeding that obtained after the secondary injection or first challenge.

Antiserum testing.—The antiserum obtained from secondary injec-

[5] Canadian M.S.E. Limited, Model 1 MSE Homogenizer.
[6] Spinco preparative ultracentrifuge, Head No. 40.

tion was tested in a number of ways. As already mentioned, CF titers against fresh preparations of antigen were determined. In addition, CF titers against whole ascites cells, their nuclei, mitochondria, and nucleoprotein were also ascertained to compare with those found earlier for antisera to other fractions. For further comparison an ascites cell agglutination titer was obtained in the same manner as was described earlier (Horn, 1956).

Finally, the cytotoxicity of this antiserum to the ascites cell was tested *in vivo* and under a variety of *in vitro* conditions.

The *in vivo* experiment was similar to earlier tests of antisera against other cell components. Immediately after receiving a measured dose of ascites cells (4.0×10^6/mouse), 20 mice were each given an intraperitoneal injection of 0.25 ml of undilute, untreated antiserum. Twenty others that received the same ascites challenge were each given 0.25 ml of untreated, normal rabbit serum (NRS).[7] Two and 4 days after this initial treatment, the 40 mice were given identical injections of respective sera. Their survival was noted twice daily for 60 days.

The *in vitro* experiments were designed for studying the effects on cytotoxicity of the three variables, cell concentration, complement, and incubation temperature.

First, a constant concentration of untreated AS and untreated NRS were incubated with different known dilutions of ascites cells for 1 hour at room temperature before their injection into mice. Twenty mice were used for each concentration, which, when injected as a 0.2 ml suspension, ranged from 8×10^6 to 0.5×10^6 cells per mouse in a twofold dilution series. Their individual survival was noted for 60 days.

In a second group of experiments the role of rabbit complement in the *in vitro* cytotoxic reaction was tested in two ways. The cytotoxicity of heat-inactivated versus untreated antiserum was compared with that of similarly treated anti-mitochondrial serum (AM), the cytotoxicity of which had already been demonstrated (Horn, 1956). Inactivation of the treated sera consisted of exposing them to 56° C for 30 minutes. Quantities of untreated and inactivated, both experimental and normal, were mixed with equal quantities of ascites cell suspensions (6.8×10^6/0.1 ml) and allowed to incubate 1 hour at

[7] The normal rabbit serum was collected and pooled from bleedings of those specific rabbits later used for the production of antisupernatant serum.

room temperature. Aliquots (3.4×10^6 cells) of each mixture were then injected into mice whose survival was noted twice daily for 60 days. A second method provided a control of the first by removing Ca and Mg ions necessary to the complement reaction. The naturally occurring complement in the NRS was determined by CF, and the amount of sequestering reagent (Versene) necessary to prevent the reaction was also determined. Twice the amount of reagent necessary to prevent the reaction was then added to the rabbit antiserum and its *in vitro* cytotoxicity tested as described for the preceding experiment.

The third experiment was designed to detect effects of as short as 15 minutes incubation at elevated temperatures of the untreated sera on the tumor cells. Since incubation of ascites cells at 37° C in air with normal rabbit serum produced an apparent cytotoxicity, the experiments were repeated in an atmosphere of nitrogen with an incubation time of 15 minutes.

Results

Complement fixation and cell agglutination activity.—Table 1 is a comparison of the CF titers of all the antisera prepared against

TABLE 1

Complement fixation titers for homologous and heterologous combinations of antisera and antigens in the separated fractions of the ascites tumor cell, in the whole cell, and in mouse ascitic fluid

	Antigens					
Antisera[a]	Cells	Nuclei	Mitochondria	Nucleo-protein	Supernatant	Ascitic Fluid
AC	320	40	160	10–20[b]	20	20
AN	80–160	160	320	80–160	10	—
AM	80–160	160–320	160–320	20	20	10
ANP	40	20–40	80	80	10	—
AS	160	40	40	10	160	10
AAF	320	40	80–160	40	40	160–320

[a] AC — whole cell antiserum; AN — nuclei antiserum; AM — mitochondria antiserum; ANP — nucleoprotein antiserum; AS — supernatant antiserum; AAF — ascitic fluid antiserum.

[b] A one-tube variation in range was not uncommon since the tests were run a number of times.

ascites cell components when tested with all the varieties of antigens used. Table 2 is a summary of the ascites cell agglutination titers obtained with the same antisera.

In vivo cytotoxicity.—Fig. 1 depicts the daily mortality of two groups of mice, one receiving ascites cells and intraperitoneal injec-

TABLE 2
Ascites Cell Agglutination Titers for Antisera Prepared Against the Ascites Cell and Its Components and Two Unrelated Antigens

Antiserum	Titer
Antiserum to whole cells (AC)	1280
Antiserum to cell nuclei (AN)	160
Antiserum to cell mitochondria (AM)	80
Antiserum to cell nucleoprotein (ANP)	0
Antiserum to cell supernatant fraction (AS)	160
Antiserum to mouse ascitic fluid (AAF)[a]	80
Antiserum to bovine plasma albumin (ABPA)[b]	0

[a] The AAF gave a homologous precipitin titer greater than 1:1000 in antigen dilution tests.

[b] The ABPA gave a homologous precipitin titer of $1:10^5$; a CF of 1:1280. It did not react precipitin-wise with the supernatant or ascitic fluid antigens.

tions of NRS, the other the same number of cells and intraperitoneal injections of AS. The mean survival of the group treated with NRS was 12.5 days, the last animals dying on the seventeenth day. On the other hand, all the animals receiving AS were still living at 15 days,

In all figures the number above each bar that terminates before 60 days represents the mean survival time of that mouse group. Each column represents 20 mice.

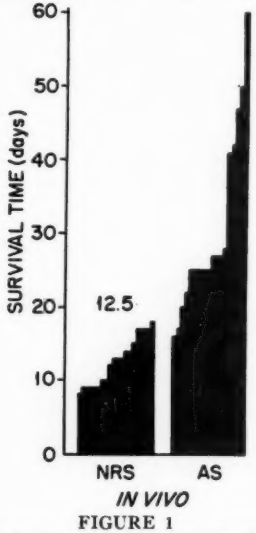


FIGURE 1
Daily survival of mice that have received a tumor implant on day 0 and three successive injections of NRS or AS.

and one animal was still alive at 60 days when the experiment was terminated.

In vitro cytotoxicity.—Fig. 2A shows the daily survival for 60 days of mice inoculated with aliquots of tumor cells pretreated with NRS, AS, and AM at room temperature for 60 minutes. The mean survival time for the NRS-treated controls was 7.4 days.

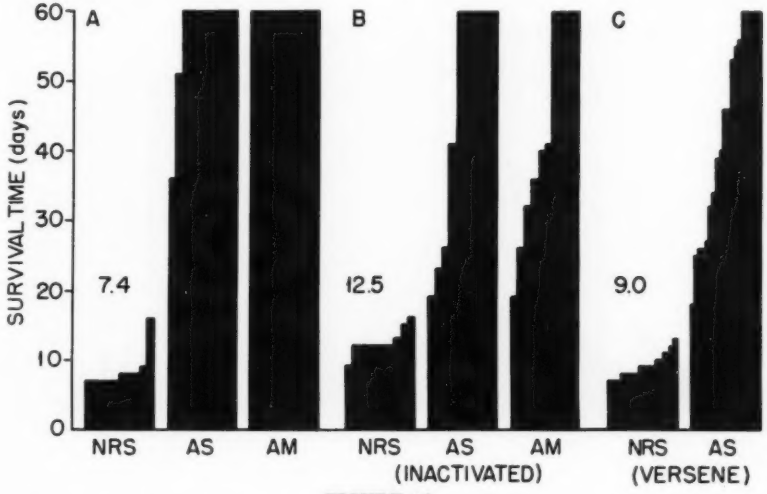


FIGURE 2A

The daily survival of mice that have received equal numbers of tumor cells treated with NRS, AS, or AM.

FIGURE 2B

Identical to 2A except that all sera were first heated to 56° C for 30 minutes before being used to treat tumor cells.

FIGURE 2C

Similar to 2A except that all sera were treated with enough chelating agent to prevent complement activity.

When the sera used to suspend the cells were first heat inactivated, these results were slightly altered (Fig. 2B), indicating the intervention in part of complement or some other heat-labile substance. Survival of animals receiving AS- or AM-treated cells was slightly poorer, although the survival of the controls improved slightly (12.5 days mean survival). If, instead of heat-treated sera, chelated (Versonated) sera were used, the result was a similar slight decrease in survival time (Fig. 2C), from which we concluded a partial minor

complement-dependent activity. The difference between mean survival times of the controls in these two experiments is of doubtful significance.

The effect of varying the concentration of cells treated with undiluted serum is shown in Fig. 3. The highest concentration of cells (8×10^6/mouse) treated with antiserum (AS 1) showed little effect from the exposure to the cytotoxic serum, but treatment of all dilutions of this cell concentration produced a measurable cytotoxic effect.

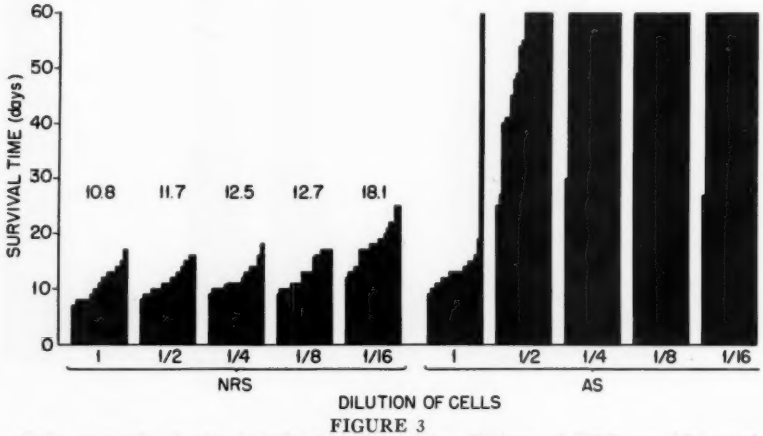


FIGURE 3

Daily survival of mice inoculated with various dilutions of NRS- or AS-treated ascites cells. AS 1 = 8×10^6 cells/mouse; AS ½ = 4×10^6 cells; AS ¼ = 2×10^6, etc.

Indeed, after two halving dilutions (AS ¼ or 2×10^6 cells/mouse) of the cell concentration, protection was essentially complete, although the controls had a mean survival time of but 12.5 days.

Increasing the temperature of cell exposure to sera from ambient (23° C) to 38° C produced the result shown in Fig. 4A. The normal serum seemed to be extremely deleterious to the tumor cells (as measured by mouse survival) if the exposure was as short as 15 minutes. When a portion of the experiment was repeated (15 minutes at 38° C) but in an atmosphere of nitrogen (Fig. 4B), the cytotoxicity of the AS emerged clearly. Comparison with Fig. 3 (AS ½) would indicate that 15 minutes' incubation at 38° is roughly equivalent to 1 hour at room temperature.

Discussion

The present results add another cytotoxic antiserum to the two already named in these continuing studies on the Ehrlich ascites tumor cell of mice. Antinuclei and antimitochondria sera prepared against components of these cells have already been reported as cytotoxic (Horn, 1956).

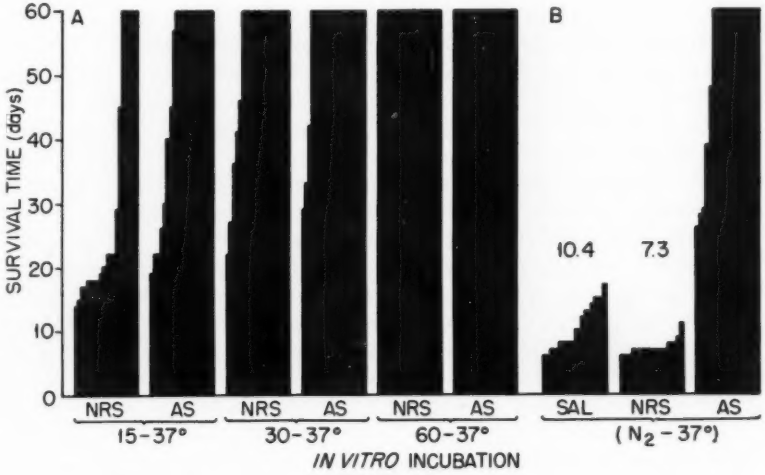


FIGURE 4A

Daily survival of mice inoculated with aliquots of tumor cells that have been incubated in air at 37° with NRS or AS for 15, 30, or 60 minutes.

FIGURE 4B

Daily survival of mice inoculated with aliquots of tumor cells that have been incubated at 37° C in an atmosphere of nitrogen with saline (SAL), NRS, or AS for 15 minutes.

The separation and use of the soluble phase of a cell type as an antigen has, unfortunately, been limited to its saline-soluble components almost exclusively. The only comparable previously reported study of the soluble phase as antigen has come from that of Colter and co-workers (1956). This group studied the cytotoxicity to tumor cells of antisera prepared against deoxyribonucleoprotein, ribonucleoprotein, and a "soluble protein" fraction prepared from a saline treated cytoplasmic extract of mortar-ground, frozen Ehrlich ascites tumor cells. They reported cytotoxic activity toward tumor cells in the antisera elicited by either nucleoprotein fraction, but the application

of their "soluble protein" antiserum to tumor cells was without pronounced cytotoxic effect. Colter has since indicated (personal communication) that the "soluble protein" fraction did elicit an antiserum capable of fixing complement in the presence of the homologous antigen. From these data one may assume that the method employed to isolate this fraction did not yield a product comparable to the soluble phase defined in the present study.

The use of a single cell type from which to derive antigens, in contrast to the whole-organ or whole-animal mince school, has obvious advantages in the search for the underlying mechanism of cytotoxicity. Nevertheless, we are still faced with the rather bleak prospect, in our ignorance of cell chemistry, of having to pluck from a heterogeneous haystack a needle of unknown dimensions and composition. Compounded with the obvious multiplicity of antigens involved with each cell fraction used for these studies is a "further complication" so succinctly put by Landsteiner (1947); namely, "that one antigenic molecular species or even one determinant group does not engender just a single uniquely defined counterpart." Presumably, different rabbit antibodies are involved in the cell agglutination, CF, and cytotoxic phenomena demonstrated herein. Heidelberger and coworkers (reviewed in 1954) demonstrated the existence in the horse of two different antibody-producing mechanisms whose stimulation is to high degree dependent on the route of antigen introduction. They also showed that the type of antibody elicited, e.g., antitoxin, precipitin, is dependent on the type of antigen. Talmage et al. (1956) showed the presence of two types of antibody differing in sedimentation rate and hemolytic efficiency in sera from rabbits injected with a single antigen.

In view of these variables it is not surprising that a comparative study of the antiserum activities examined here (agglutination, complement fixation, and cytotoxicity) show no apparent correlation. There is then no basis for predicting the measure of one activity from that of another. This has been amply demonstrated, for example, by the antiserum elicited by whole-cell injections. This antiserum can agglutinate suspensions of tumor cells in high dilution, but the metabolism of enough of these same cells has certainly not been affected to such an extent that the survival of a significant number in a mouse host is prevented; this demonstrates, we feel, the absence or small quantity of cytotoxic antibody or antibodies. Tyler and Brookbank (1956), working with antisera produced against presumably "highly

pure" fertilizin, noted sperm agglutination as well as cleavage-blocking activity. Absorption of the sera with sperm removed the agglutinin with only a partial depression of the cytotoxic titer. One must conclude that either the fertilizin preparation contained at least two species of molecules, or that this is simply another example of a single antigen eliciting at least two species of antibodies.

A review of the most recent literature dealing with the possible role of complement in the cytotoxic reaction reveals clearly that a host of antigens are stimulating the production of cytotoxic sera, all of which seem to exhibit a degree of specificity to the eliciting tissue or whole animal. Using macerated Brown-Pearce carcinoma to provide cell suspensions for assay and the sera of rabbits with regressed tumors to supply the antibodies, Kalfayan and Kidd (1953) studied the cytologic changes in the exposed cells. The cytologic changes which they described were dependent on the presence of complement as well as on the antiserum. *In vitro* exposure of tumor cells to antisera produced a cytotoxic effect assayed by tissue transfer to fresh rabbit hosts; this, too, was complement dependent.

In an antiserum prepared against ruptured HeLa cells, Mountain (1955) distinguished subjectively an agglutinating from a lysing activity on these cells when exposed in tissue culture to the experimental sera. She lumped these phenomena together, unfortunately, in describing what she characterized as the cytopathogenic effect. In a test to determine complement dependency of the effect, however, she assumed that the two activities were separable since the agglutinin appears to be complement independent while the lysin is dependent on the presence of complement. In view of our results with the agglutinating ability of the whole cell antiserum, which lacked cytotoxicity (and it may be added, was complement independent), it would be interesting to know if the HeLa agglutinin is really *cytotoxic*. Agglutination alone is certainly not a definitive criterion of cytotoxicity.

Flax (1956) collected a cytotoxic antiserum from rabbits by presenting them with Ehrlich ascites tumor cells through six portals simultaneously. In light of Heidelberger's findings (op. cit.) on the relation of antibody quality to antigen route, we can consider this antiserum only superficially comparable to our whole-cell serum. Using the gamma globulin fraction of his serum, he demonstrated a complement-dependent cytotoxicity based on mouse survival, on vital

staining of exposed cells, and on enzyme studies. Miller and Hsu (1956), using homogenized HeLa cells as their antigenic stimulus, obtained cytotoxic sera from rabbits and roosters. Interestingly, the rabbit antiserum proved to be complement independent for the cytotoxic effect, but the avian serum lost a good part of its activity upon heat inactivation. In our experience with chicken serum, we have observed inevitably a heavy precipitation after short-term (48 hr) refrigeration; this suggests further basis for caution in interpretations from avian serum.

Schrek and Preston (1957) investigated the cytotoxic action on tumor cell suspensions of the sera of rats that had experienced a regressed Bagg tumor. Differential staining of the cells after exposure to the rat sera supplied their method of assay. In their experiments the cytotoxic effect was completely complement dependent. Furthermore, contact with Bagg tumor cells or with "tumor extract" (from their description of the preparative method probably a mitochondria and microsome fraction) removed the cytotoxic activity.

Among the studies of cytotoxic antisera applied to embryonic materials, there seems to have been little attempt to analyze possible complement dependency. One of the earliest studies of this type was that of Parkes (1946) who used an antiserum prepared against minced tadpoles. The antiserum proved lethal to tadpoles and dependent on complement for this effect. Ebert (1950) used heat-inactivated sera throughout his work, thus demonstrating the complement independence of the reaction. Clayton (1953) also used heat-inactivated sera to produce cytotoxic effects on exposed embryos. The use of complement-bolstered sera in both cases might have provided interesting comparative material.

From the foregoing observations the evidence seems to be overwhelming that the variety of cytotoxic effects reported in the literature are from a variety of antibodies that need have no more in common in their mode of action than that they all produce the same end point, namely, death to some restricted cell type exposed to them. The present studies, in which we have attempted to reduce the heterogeneity of possible antigens to a reasonable level by removing all nuclear and particulate material, indicate that we are dealing with at least two cytotoxic entities in the AS, the one more potent and complement independent, the other less active and complement dependent. Our observations here and in earlier work indicate that neither is agglutinating or lysing in action.

It is important to point out a difference between the AS produced for these studies and a number of cytotoxic sera produced for comparable studies where less restricted portions of cells were used (Ishikura et al., 1950; Motoyama et al., 1952; Nungester and Fisher, 1954; Mountain, 1955; Flax, 1956; Miller and Hsu, 1956). All of these investigators reported that their antisera were toxic in some degree to normal mouse tissue. The AS apparently lacked this toxicity. For economic reasons the experiments reported here were terminated at 60 days. In some instances, however, limited numbers of test animals injected with treated tumor cells were kept much longer. Their survival seemed (subjectively) comparable to that for the normal colony for their age group. The *in vivo* experiments, in which immediate toxic effects should have been most evident, seem to be the best demonstration of this lack of toxicity. One cannot be so conclusive about the possible long-range (beyond 120 days), more insidious effects on organs, e.g., kidney, liver, spleen.

The widespread cross reactivity demonstrated by the complement-fixing ability of the various antisera, together with the variety of heterologous antigens, cannot be attributed solely, or even in large measure, to the use as antigen of grossly contaminated cell fractions (Horn, 1956). For example, whole cells, nuclei, mitochondria, or microsomes could not be present in the supernatant fraction prepared as described. Anderson (1956) has amply demonstrated the lack of insoluble particulate matter in similar preparations from rat liver cells. The cross reactions, however, between AS and the whole cells or to a lesser extent with their mitochondria or nuclei are clearly shown in Table 2. This finding continues to support an earlier suggestion (Horn, 1956), namely, the universal distribution within a cell's components of common antigenic substance or substances. Unreported results from systematic absorptions of these antisera with whole cells or with mitochondria show a gradual decline in their complement-fixing ability, whether the antigen is homologous or heterologous. We further suggest now that this common antigen or antigens might be a (or the) structural protein of the membranous portions of the cell. The rapid accumulation of morphological data on the ultrastructure of cells from electron microscopy reveals in striking fashion what now appears to be the general and fundamental lamellate architecture of the nuclear membrane (Bairati and Lehmann, 1952), the Golgi apparatus (Dalton and Felix, 1954), the mitochondria (Palade, 1952),

and even the ground substance—more appropriately named, by some at least, the endoplasmic reticulum (ergastoplasm) (Dalton, 1951; Palade, 1952). A protein component of these organelles has generally been assumed from fixation studies, as has a lipid portion. Quite obviously—assuming within a cell type identity of protein in the structures named—a protein as widely distributed in the cell as this must be would play a prominent role in the economy of the cell and in turn would be one of the commonest antigens in almost any fraction of that cell. It seems a safe assumption that a good deal of this same protein would necessarily be found in the soluble phase of the cell in preparation for its withdrawal from the "pool" to permit its subsequent addition to any one of the host of "membranes" now known to exist. Such a suggestion, if perhaps naive and over-simplified, offers a working hypothesis, nonetheless, for further experimentation on the cytotoxic phenomenon. Indeed, Anderson (personal communication) from his work on the soluble phase of the rat liver cell has suggested that the spindle protein or proteins might also be common to such a postulated system. Herein one has the basis then for a rather direct approach to the problem of cytotoxicity.

The work of Stern and Elek (1957) may be particularly pertinent at this point. In a careful study of the antigenic basis for the three Cowan types of *Staphylococcus pyogenes,* these investigators prepared antisera to whole cells, cell walls, and endoplasm from the three classical types. Agglutination, precipitin, and agar diffusion studies combined with selective absorption indicated no antigenic differences between the outer and inner surfaces of the cell wall. Furthermore, extracts from whole cell walls showed complete reactivity among the three types. The antisera to the endoplasmic fractions showed a great deal of cross reactivity among the three types, although agar diffusion studies indicated the possibility of minor groups and type-specific factors. Unfortunately, there was no indication of whether the endoplasm and cell wall shared common antigens. In conclusion, Stern and Elek decided that the cell surface possessed the major and minor antigens important to the typing reaction investigated. The major antigens provide the group specificity (cross reacting with all types), but the minor are type specific and type shared.

Provided that these results of Stern and Elek may be applied in a broad sense to the present problem dealing with quite different material, a further basis is suggested for a universality of structural cellu-

lar protein and an explanation is provided for any specificity that may be proved in the future. Finally, their results provide in a convincing manner the basis for at least some of the cross reactivity so easily demonstrated in these studies.

Summary

An antiserum was prepared against the soluble phase of Ehrlich ascites tumor cells, which possesses a high cytotoxic activity against these cells whether applied *in vivo* or *in vitro*. A major portion of this activity is complement independent, a minor part complement dependent. A comparison of tumor cell agglutination and complement fixation activities for five antisera to ascites cells and their components is presented to demonstrate the impossibility of predicting cytotoxic activity from these tests. An interpretation of the wide serological cross reactions demonstrated by the complement fixation tests is given, together with a working hypothesis to explain the cytotoxicity reaction in this case.

Acknowledgments

This work has been conducted in the laboratory of Dr. Norman G. Anderson. To him and to Dr. Takashi Makinodan go my especial thanks, particularly for their stimulating encouragement and interest in the problem as well as their continual aid both material and mental. I am also grateful to Miss Mary Elizabeth House for her capable assistance in handling a large portion of the laboratory work.

References

1. Anderson, N. G. 1956. Studies on isolated cell components. IX. The soluble phase. *Exptl. Cell Research*, **11**, 186-196.
2. Bairati, A., & Lehmann, F. E. 1952. Über die submikroskopische Struktur der Kernmembran bei *Amoeba proteus*. *Experientia*, **8**, 60-61.
3. Burke, V., Sullivan, N. P., Peterson, H., & Weed, R. 1944. Ontogenetic change in antigenic specificity of the organs of the chick. *J. Infectious Diseases*, **74**, 225-233.
4. Clayton, R. M. 1953. Distribution of antigens in the developing newt embryo. *J. Embryol. and Expt. Morphol.*, **1**, 25-42.
5. Colter, J. S., Koprowski, H., Bird, H. H., & Pfeister, K. 1956. Immunological studies with protein fractions isolated from Ehrlich ascites carcinoma cells. *Nature*, **177**, 994-995.
6. Dalton, A. J. 1951. Electron micrography of epithelial cells of the gastrointestinal tract and pancreas. *Am. J. Anat.*, **89**, 109-134.
7. ———, & Felix, M. D. 1954. Cytologic and cytochemical characteristics of the Golgi substance of epithelial cells of the epididymis *in situ*, in homogenates and after isolation. *Am. J. Anat.*, **94**, 171-208.

8. DULANEY, A. D., & ARNESEN, K. 1949. Cytotoxic action of antisera to cell components of normal and leukemic mouse spleens. *Proc. Soc. Exptl. Biol. Med.*, **72**, 665-668.

9. ———, GOLDSMITH, Y., ARNESEN, K., & BUXTON, L. 1949. A serological study of cytoplasmic fractions from the spleens of normal and leukemic mice. *Cancer Research*, **9**, 217-221.

10. EBERT, J. D. 1950. An analysis of the effects of anti-organ sera on the development, *in vitro*, of the early chick blastoderm. *J. Exptl. Zool.*, **115**, 351-378.

11. FLAX, M. H. 1956. The action of anti-Ehrlich ascites tumor antibody. *Cancer Research*, **16**, 774-783.

12. FLICKINGER, R. A., & NACE, G. W. 1952. An investigation of proteins during development of the amphibian embryo. *Exptl. Cell Research*, **3**, 393-405.

13. HEIDELBERGER, M. 1954. The precipitin reaction and the studies of native and denatured proteins and derivatives. In, Serological Approaches to Studies of Protein Structure and Metabolism, W. H. Cole, ed. Rutgers University Press, New Brunswick, N. J., pp. 10-22.

14. HORN, E. C. 1956. Ascites tumor development. II. Cytotoxicity of various antisera prepared against Ehrlich ascites tumor cell components. *Cancer Research*, **16**, 595-599.

15. ISHIKURA, H., ITAKURA, S., USUBUCHI, I., SUZUKI, K., AIZAWA, M., IMAMURA, T., & MORI, S. 1950. On influence of anti-Yoshida sarcoma sera of healed rats and sensitized rabbits upon neutralization, passive immunity and therapy of Yoshida sarcoma. *Gann*, **41**, 177-179.

16. KALFAYAN, B., & KIDD, J. G. 1953. Structural changes produced in Brown-Pearce carcinoma cells by means of a specific antibody and complement. *J. Exptl. Med.*, **97**, 145-162.

17. LANDSTEINER, K. 1947. The Specificity of Serological Reactions. Harvard University Press, Cambridge, Mass., p. 144.

18. MALMGREN, R. A., BENNISON, B. E., ANDERSON, B. F., & RISLEY, C. C. 1951. A serologic study of the microsome fraction of normal and neoplastic mouse tissues. *J. Natl. Cancer Inst.*, **11**, 1277-1286.

19. MILLER, D. G., & HSU, T. C. 1956. The action of cytotoxic antisera on the HeLa strain of human carcinoma. *Cancer Research*, **16**, 306-312.

20. MOTOYAMA, T., TOZAWA, T., AIZAWA, M., & IMAMURA, T. 1952. The therapeutic effect upon Yoshida sarcoma of immune sera against Yoshida sarcoma of rabbit absorbed with several methods. *Gann*, **43**, 248-250.

21. MOUNTAIN, I. M. 1955. Cytopathogenic effect of antiserum to human malignant epithelial cells (strain HeLa) on HeLa cell culture. *J. Immunol.*, **75**, 478-484.

22. NUNGESTER, W. J., & FISHER, H. 1954. The inactivation *in vivo* of mouse lymphosarcoma 6C3HED by antibodies produced in a foreign host species. *Cancer Research*, **14**, 284-288.

23. PALADE, G. E. 1952. A study of fixation for electron microscopy. *J. Exptl. Med.*, **95**, 285-298.

24. ———. 1952. The fine structure of mitochondria. *Anat. Record*, **114**, 427-452.

25. PARKES, A. S. 1946. Anti-tadpole sera. *Nature*, **157**, 164-165.

26. SCHREK, R., & PRESTON, F. W. 1956. Toxicity of homologous immune serum to a transplantable tumor; studies using phase microscopy and cinemicrography. *J. Natl. Cancer Inst.*, **16**, 1021-1045.

27. ———, & PRESTON, F. W. 1957. Cytotoxicity of a homologous immune serum to a transplantable rat tumor by the method of unstained cell counts. *Cancer Research*, **17**, 102-107.

28. STERN, H., & ELEK, S. D. 1957. Antigenic structure of *Staphylococcus pyogenes*. *J. Pathol. and Bacteriol.*, **73**, 473-483.

29. TALMAGE, D. W., FRETER, G. G., & TALIAFERRO, W. H. 1956. Two antibodies of related specificity but different hemolytic efficiency separated by centrifugation. *J. Infectious Diseases*, **98**, 300-305.

30. TYLER, A., & BROOKBANK, J. W. 1956. Antisera that block cell division in developing eggs of sea urchins. *Proc. Natl. Acad. Sci. U. S.*, **42**, 304-308.

31. ———, & BROOKBANK, J. W. 1956. Inhibition of division and development of sea urchin eggs by antisera against fertilizin. *Proc. Natl. Acad. Sci. U. S.*, **42**, 308-313.

Growth, 1957, **21**, 239-261.

OOGENESIS IN ADULT *DROSOPHILA MELANOGASTER*. IV. HEREDITARY OVARIAN TUMORS[1]

R. C. KING, R. G. BURNETT AND N. A. STALEY

Northwestern University, Evanston, Illinois

(Received September 5, 1957)

INTRODUCTION

THERE are many instances where genes have been implicated in the production of uncontrolled cell division. The gene *polymitotic* in maize (Beadle, 1931) is a classic example of a gene which produces supernumerary cell divisions *without* chromosomal duplication. Conversely, mutants which prevent cytokinesis are known (Beadle, 1932). Mutants exist which permit division of accessory sperm nuclei (Whiting, 1943; Rothenbuhler and Gowen, 1955). Supernumerary cell divisions which arise in interspecific hybrids during spermatogenesis have been described by Dobzhansky (1934) and Crew and Koller (1936). Tumors of species hybrids (presumably resulting from genic imbalance) have been described in mammals (Little, 1939), fish (Gordon, 1953), and in plants (Satina, Rappaport and Blakeslee, 1951 and Kehr and Smith, 1954). Different related species and the various races of a single species differ radically in cancer proneness, and such differences have in many cases a genetic basis (Huxley, 1956). In man certain rare tumorous conditions (xeroderma pigmentosum, retino-blastoma, multiple intestinal polyposis, and multiple neurofibromatosis) have shown single factor inheritance (Reed, 1955). In the mouse the inheritance of mammary tumors, lung tumors and of leukemia has been worked out in certain instances (Little, 1947 and Gruneberg, 1952). There is evidence from tissue culture studies (Puck and Fisher, 1956) that the cell population of the HeLa human cancer is genetically non-homogeneous. The parallelism between the mutagenicity and carcinogenicity of certain agents implicates gene mutation as a factor in the production of neoplasia (Muller, 1954). It has been argued that the existence of virus-induced tumors rules out mutation as a

[1] Research supported by the U. S. Atomic Energy Commission (Contract No. AT (11-1)-89, Project 12), the National Science Foundation (research grant NSF-G4816) and by the Graduate School of Northwestern University.

factor in carcinogenesis. It is now known, however, that certain viruses can reside on host chromosomes, and the possibility exists that such viruses can modify adjacent host chromatin and so produce a reversible "mutational" state.

The gene *fused* of *Drosophila melanogaster* produces ovarian tumors (King and Burnett, 1957) and thus belongs in the catalogue of mutant genes implicated in the production of abnormal cell division.[2] *Fused* belongs to the recessive female sterile category of mutations. A subsequent cytological study of 21 other mutations of this category added two more mutants (*female-sterile* and *narrow*^{2}) to the group characterized by ovarian tumors.[3] Once one takes into consideration the anatomical differences between the human and the fruit fly ovary, the *Drosophila* tumors to be described show some resemblance to those rare benign, human, ovarian neoplasms called Brenner tumors. However, at this early stage it is perhaps wise to suspend judgement as to the relation between *Drosophila* ovarian tumors and tumorous growths in mammals. The data to be reported provide a more precise description of *female-sterile, narrow*^{2} and *fused* than was available previously.

Material and Methods

Information as to the history of the mutants, the stocks used and their source are presented in Table 1. Generally flies were grown on medium (for formula see King and Wood, 1955) in half-pint milk bottles at 23-25° C. The method used for egg collections has been described previously (King, 1955). Large hoops (7.6 cm o.d.) were used. The initial fly population was 10 females (the genotype varied according to the experiment) and 20 *Oregon R*-wild type males. Females were allowed to lay continuously and hoops were transferred to fresh medium daily. The medium in Petri dishes was made according

[2] Whether the melanotic "tumors" of *Drosophila melanogaster* should be included is doubtful (Wilson, King and Lowrie, 1955). Rizki (1957) has shown that in certain pseudotumorous strains the "tumors" result from encapsulation during the *larval* stage of various tissues by lamellocytes. Normally lamellocytes are not found in the blood in large numbers until the *pupal* stage when a transformation of podocytes to lamellocytes takes place. However, this transformation occurs precociously in "tumor" strains. Therefore "tumors" are the result of abnormalities in metamorphosis rather than of uncontrolled cell division. It is up to those workers who consider melanotic tumors in their *Drosophila* strains to be true tumors to show that the above phenomenon is not the mechanism in their material.

[3] Negative results were obtained with the following mutants: chromosome 1; *deep orange, stubarista, diminutive, singed*^{36a}, *ocelliless, lozenge*^{8}, *raspberry*^{4}, *tiny, narrow-abdomen, rudimentary*^{9}; chromosome 2; *rotund, apterous*^{4}, *apterous*^{56f}, *female-sterile 2.1, combgap, minus;* chromosome 3; *crumpled, brief;* and chromosome 4; *shaven-depilate.*

TABLE 1
Genetic Material

Locus	Symbol	Name	Discoverer	Date	Occurrence	Stock	Source
1–59.5	fu	fused	C. B. Bridges	Nov. 4, 1912	spontaneous	fu/M5	Yale
	fu^{ff}	fused-formalin food	C. Auerbach	Feb. 1952	formalin-induced	fu^{ff}/ClB	U. of Utrecht
	fu^{57a}	$fused^{57a}$	C. E. Purdom	Jan. 1957	azo-mustard-induced	fu^{57a}/M5	Pollard's Wood Research Station, England
	fu^{57f}	$fused^{57f}$	C. E. Purdom	June 1957	azo-mustard-induced	fu^{57f}/M5	Pollard's Wood Research Station, England
2–5 ±	fes	female-sterile	C. B. Bridges	Mar. 25, 1929	spontaneous	fes Alu lt/al^2 Cy lt^3	Indiana U.
2–83±	nw^2	$narrow^2$	F. Payne	Dec. 5, 1916	spontaneous	nw^2/Cy RNS	California Inst. of Technology

to the formula (5g sucrose, 20g agar, 2g powdered charcoal, 5 ml propionic acid, 1000 ml water). Flies fed on this medium or on this medium to which a drop of a thick suspension of living *Saccharomyces cerevisiae* (Fleischmann's active dry yeast) was added.

Feulgen stained whole mounts were made of ovaries of various ages and various genotypes.[4] Observations were made with a number 5 American Optical research microscope equipped with an Ortho-illuminator. Photographs were taken using Pola Pan 200 film mounted in a Polaroid Land camera back attached to an American Optical photomicrographic setup.

Phenotypic characterization of *FUSED*

External Morphology

The mutation *fused* is an example of a gene with manifold effects. *Fused* flies are characterized by abnormal wings which are generally held outstretched (in the case of *fu* and fu^{57f}), but may be folded over the back (fu^{ff} and fu^{57a}). The pattern of longitudinal wing veins is modified in that veins 3 and 4 are fused from their origin to beyond the anterior crossvein (which is therefore eliminated). Fusions between L 3 and L 4 may occur more distally with the result that the first posterior wing cell may be divided into a series of compartments. In the case of fu^{57f} veins 3 and 4 are often completely fused. In all alleles the ocelli are reduced in number or are entirely absent, and the ocellar and anterior scutellar bristles are also reduced in number or are entirely absent. The length of the scutellum is generally reduced.

Miscellaneous characteristics

Fused females eclose about 3 days later than do heterozygous females or *fused* males. The longevity of *fused* females is considerably shortened (cf. figure 1).

The fecundity of *fused* females is about 7% that of fu/+ females

[4] Procedure: Place etherized female fly in depression slide; cover with *Drosophila* Ringer solution; dissect out ovaries using jeweler's forceps. After twenty or so ovaries have been collected drop them into a small vial containing one normal HCl at 60° C; maintain at 60° C for 14 minutes. To change solutions gently pour out fluids; use a clean micropipette to squirt in each new solution; handle tissue gently. Stain in leucobasic fuchsin until apical portions of ovary take on a deep violet color (5-60 minutes). Next two rinses (2 minutes each)—water saturated with SO_2, 10% ethanol, 50% ethanol, 95% ethanol, 100% ethanol, ethanol-xylene (1:1), and finally xylene. Transfer stained ovaries in a drop of xylene to a clean microscope slide. Examine at 20X using a stereoscopic microscope. Tease ovary into its constituent ovarioles using jeweler's forceps or glass micro-needles. Put a drop of permount over the tissue and cover with a cover slip.

(Figure 2). There are at least three reasons for this. The first is the smaller number of oöcytes per ovary (approximately ½ that found in *fu* heterozygotes) which is due to the smaller number of ovarioles (Table 2). The second and third reasons are that many contigu-

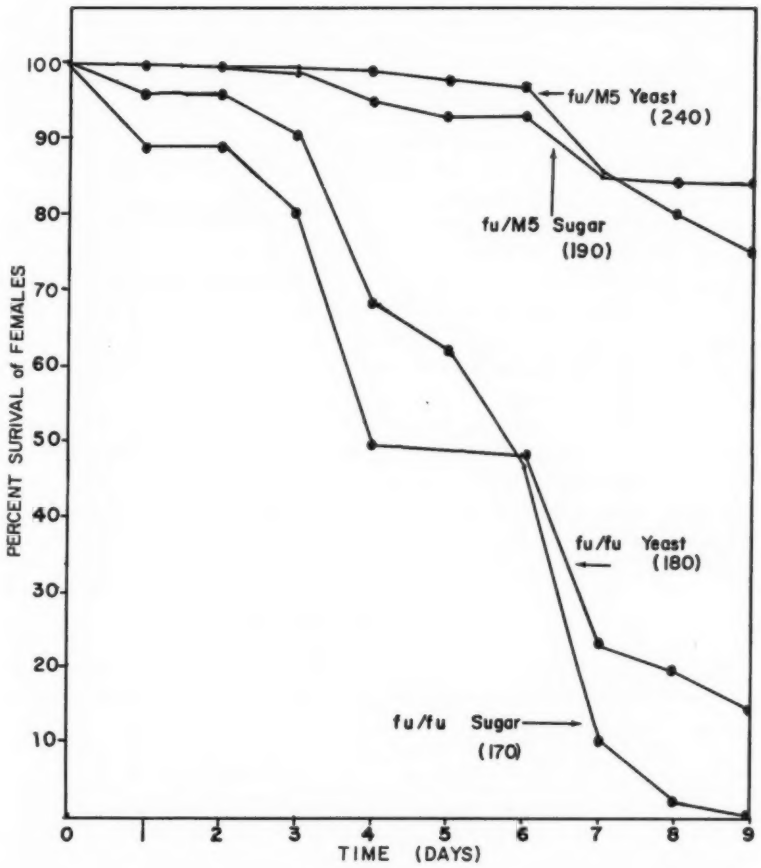


FIGURE 1

Longevity of adult female *Drosophila melanogaster* as a function of genotype and nutrition. Numbers in parentheses give the number of females at the start of the experiment.

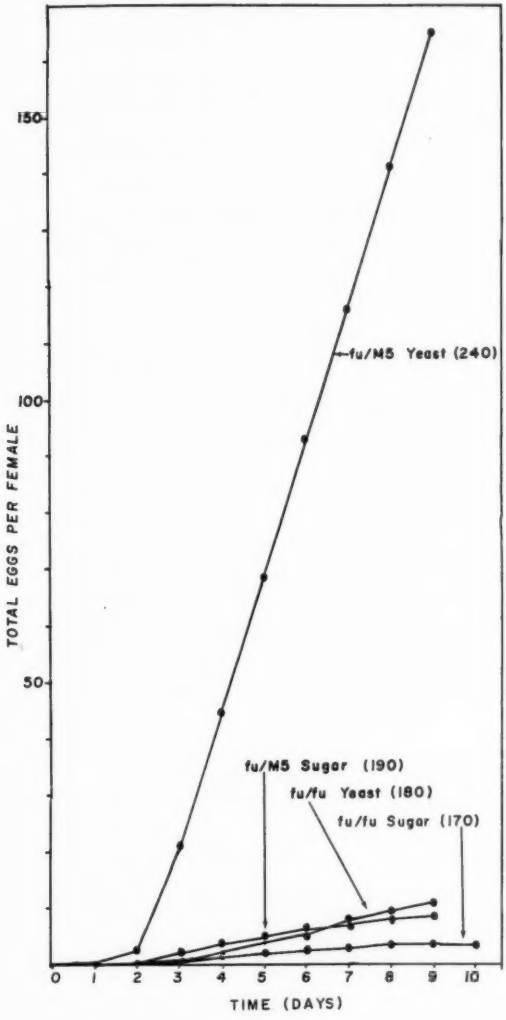


FIGURE 2

Fecundity as a function of genotype and nutrition. Numbers in parentheses give the number of females at the start of the experiment.

ous developing egg chambers undergo fusion, while others are converted into tumors.[5]

TABLE 2
The Effect of *fused* on Ovary Size

Genotype	Number of ovaries observed	Average number of ovarioles/ovary	Average number of oocytes/ovariole	Average number of oocytes/ovary
fu/fu	12	8.7	7.5	65.3
fu/M5	7	15.3	7.7	117.8

The few normal appearing eggs which are laid produce adults only if they have been fertilized by sperm bearing the + allele of *fused* (Lynch, 1919). It follows that these apparently normal eggs are deficient in some vital substance (hereafter called the *fused*+ substance) that can be supplied by the + allele subsequent to fertilization. *Fused* eggs (from *fused* females) fertilized by *fused* or Y-bearing sperm develop into embryos which suddenly become abnormal at 5 to 5½ hours after fertilization (Counce, 1956). There is established a general asymmetry in germ layers which is responsible for many ensuing aberrations. Such embryos never emerge, but manage to remain alive long after normal embryos have emerged as larvae. *Fused* eggs from heterozygous *fused* females develop normally irrespective of the genotype of the sperm which fertilizes them. One simple explanation for this maternal effect is that quantities of the fu+ substance sufficient for normal embryogenesis have been incorporated into the yolk by nurse cells (which have the maternal genotype and consequently the fu+ allele).

The Tumors of *fused* ovaries

Tumors occur in the ovaries of *fu*, fu^{ff}, fu^{57a} and fu^{57f} females. Low power photomicrographs of characteristic preparations are shown in Figures 3 and 4; higher power photomicrographs in Figures 5 and 6. A tumor consists of a group of between 100 and 10,000 cells (each 5-8 micra in diameter with nuclei similar in size to oogonia) surrounded by a follicular epithelium. Numerous mitoses are observed in such tumors (Figures 7, 8 and 9). Generally the karyotype appears normal, but aneuploid metaphases and ring chromosomes have been observed. The tumors are often characterized by centrally located

[5] The reproductive system of *fused* females contains normal-appearing spermathecae, a ventral receptacle and accessory glands.

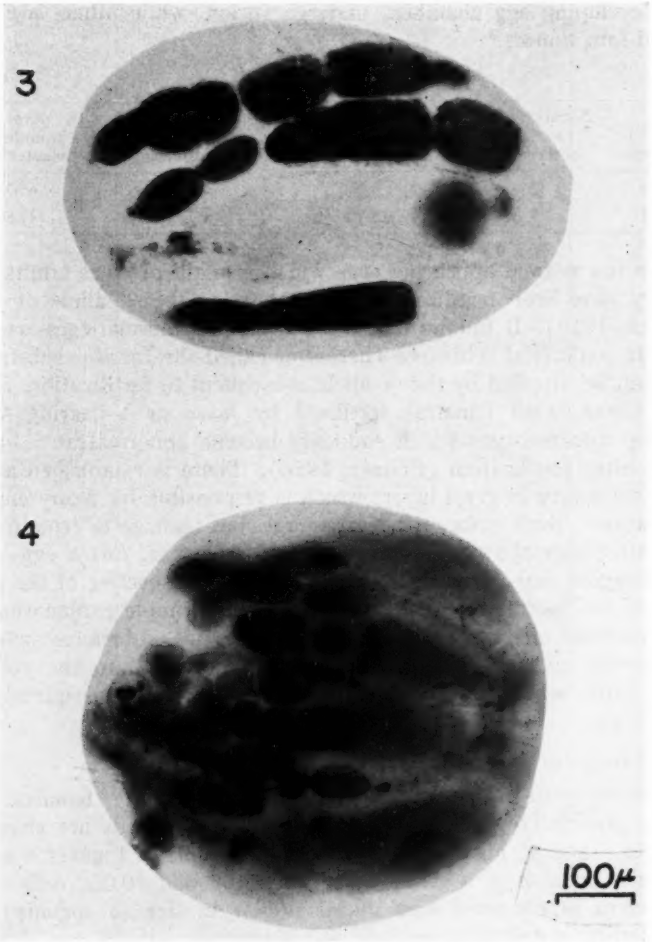


FIGURE 3

Three ovarioles plus a stage 14 oocyte from a 10 day *fused* female. 14 Tumorous chambers are present. The cylindrical tumor in the lower ovariole originated from the fusion of several tumorous chambers.

FIGURE 4

A whole mount of a single ovary from a 10 day *fused* female. Note that among the immature oocytes normal chambers are in the minority (3 normal stage 6 oocytes/~ 15 tumors).

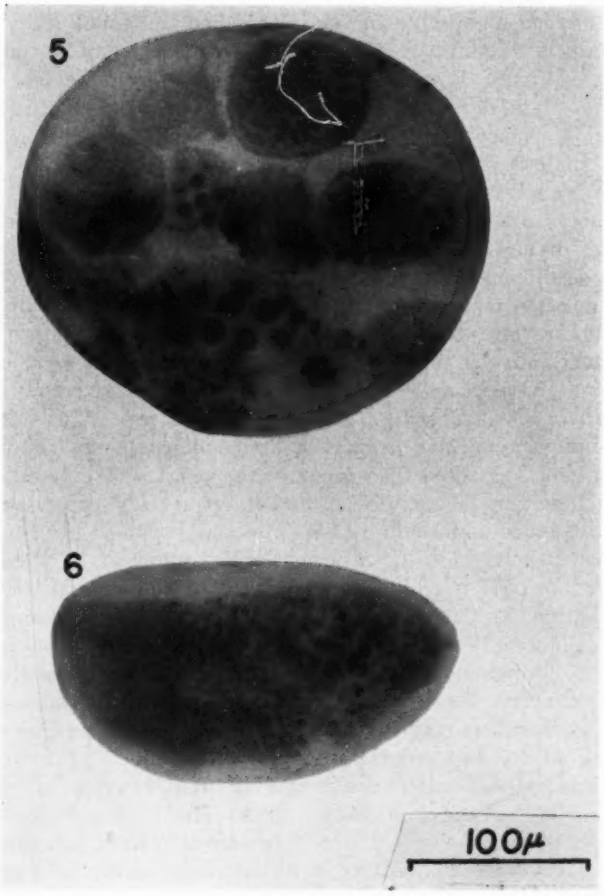


FIGURE 5

Tumors in the ovary of a 13 day *fused* female. Note the well defined follicular epithelium surrounding the tumor and the centrally located clusters of Feulgen positive granules.

FIGURE 6

One of the largest tumors observed (containing approximately 8,000 cells). 14 day *fused* female.

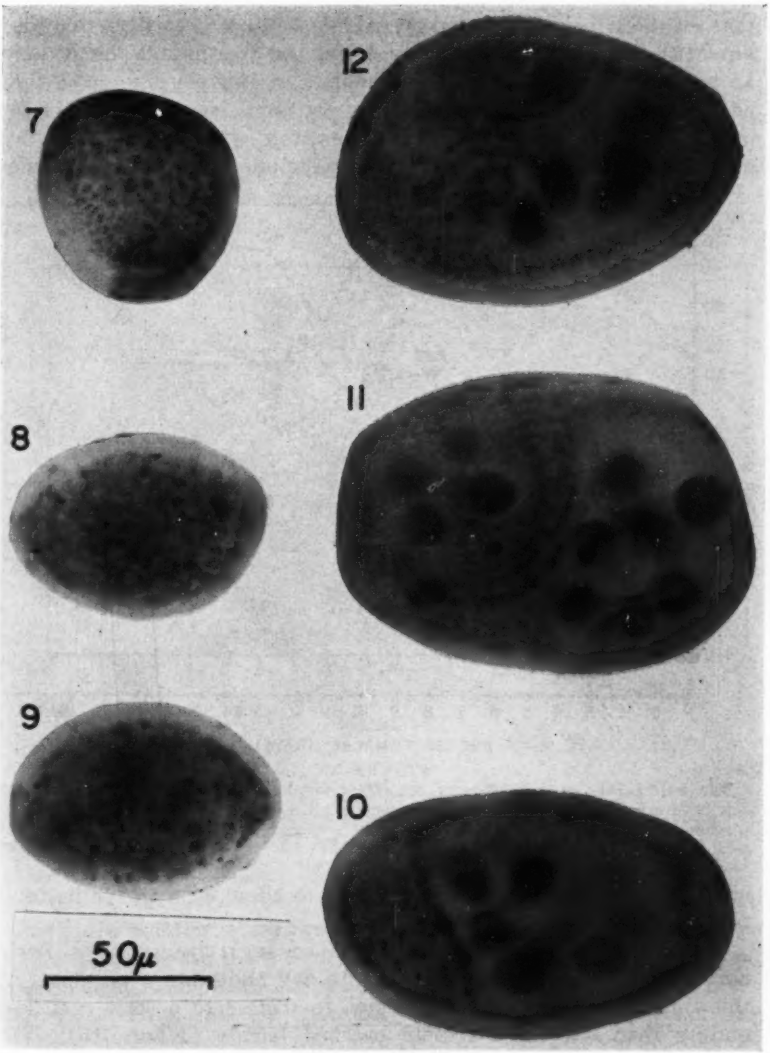
clusters of Feulgen positive granules (Figure 5). Ovarioles are often found in which a normal chamber is preceded and followed by tumorous chambers (Figure 4). In other cases ovarioles may be completely tumorous (Figure 3) or completely normal. Often the two ovaries may differ markedly in size. One ovary may have 10 times the mass of the other, because the larger ovary contains some stage 14 eggs, whereas the smaller one has nothing but tumors and immature oocytes (none more advanced than stage 7).

Chambers are often observed which contain a heterogeneous assemblage of cells (some resembling oogonia; others, nurse cells at various developmental stages; and others resembling oocytes). Such chambers often result from the fusion of various numbers of normal and tumorous chambers. Fusions of contiguous chambers are a common abnormality in various mutant strains of *Drosophila melanogaster*. We have observed such fusions in flies homozygous for the genes *diminutive, rotund, ocelliless, female sterile 2.1, raspberry*^{4}, *minus* and especially in *crumpled,* and *combgap* (also reported by Beatty, 1949). Fusions also occur in the ovaries of flies exposed to ionizing radiation (King, 1957a) and in *lgl* ovaries transplanted into *fes* hosts (Gloor and Hadorn, 1942).

In cases where fusion of a normal and a tumorous chamber takes place, tumor cells may invade the tissue of the normal developing egg (Figures 10, 11 and 12). As the flies age the frequency of tumors (Figure 13) and the frequency of fusions (Figure 14) increase. Fusions of contiguous chambers do not occur at an elevated frequency until the 6th day. Fusions generally occur only in tumorous ovarioles. Tumorous chambers may fuse together or normal and tumorous chambers may adhere, but generally two normal chambers never fuse. By day 20 it is possible only to arrive at a minimum estimate for the number of tumors and the number of fusions. This is because it is impossible to decide in the case of many tumorous masses just how many fusions have occurred. Therefore the declines shown in Figures 13 and 14 at day 20 may not be real.

Effect of nutrition on longevity, fecundity and ovarian cytology.

A comparison was made of the longevity, ovarian development and fecundity of *fu* homozygotes and heterozygotes fed on sugar-agar and on sugar-agar supplemented with live yeast. The longevity of female flies fed on the supplemented medium is consistently higher than



FIGURES 7-12

From a 10 day *fused* female

(7, 8, 9) Tumorous chambers showing metaphases. In all 12 are shown clearly. (10) An early stage in the fusion of a normal and a tumorous chamber. Note the wall separating the two chambers. (11 and 12) Normal chambers being invaded by tumorous cells. In 11 a portion of the wall separating the two types of tissue is still present. In 12 all traces of it have disappeared.

that of flies fed on sugar-agar alone (Figure 1). Heterozygous (fu/M5) females fed on yeast lay during the 3rd through the 9th day an average of 24 eggs per day (Figure 2). Homozygous *fused* flies on the other hand lay about 1.7 eggs per day. Sugar-fed, *fu*/M5 females lay approximately 8 eggs and then stop; whereas *fu* females lay only about 3 eggs. It appears that under our conditions the adults

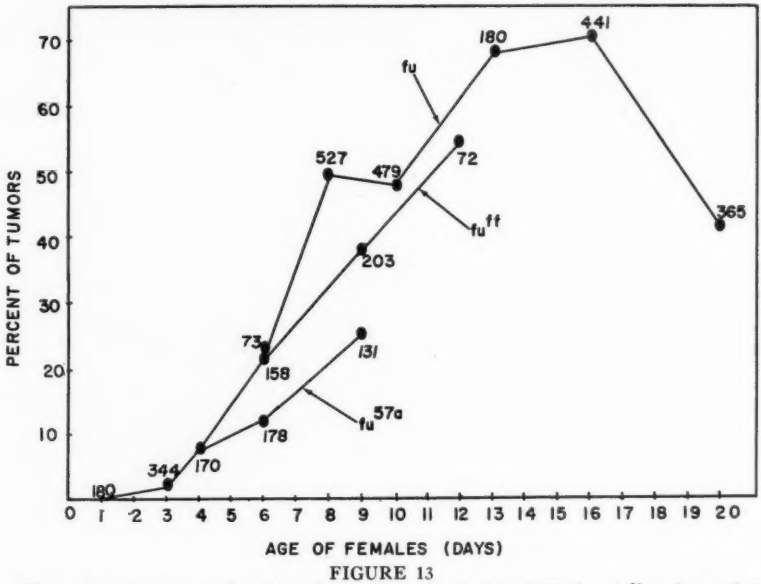


FIGURE 13

Tumor frequency as a function of age. Data for 6, 9 and 12 day fu^{ff} and 6 and 9 day fu^{57a} are included with those for *fu* females. The tumor incidence of fu^{57a} females appears to be lower than that of *fu* and fu^{ff} females. The number above each point gives the number of developing eggs examined.

have only sufficient larval food reserves to allow them to synthesize a few eggs.

The ovaries of *fu*/M5 females fed on sugar-agar show a fairly normal stage distribution up to the 8th day under our conditions. At this time, however, the ovary begins to regress to a state even less mature than that of a freshly hatched female (Figure 16). The ovaries of 13 day flies fed sugar-agar contain no stage 7 oocytes (Figure 15); whereas 7's are plentiful in the freshly hatched female.

It follows that the metabolism of stage 6 and 7 oocytes differs, and that the sugar-agar medium lacks one or more nutrients necessary for the further development of stage 6 chambers. Yeast-fed, 13 day old, *fu*/M5 females have ovaries which are relatively immense (Figure 17).

The germaria of flies fed for 8-13 days on sugar-agar are markedly abnormal (Figure 18 vs. Figure 19). They are spherical rather than sausage-shaped, contain no germarial cysts, show no mitoses, and contain little cellular detail. On the other hand stages 2-6 appear fairly

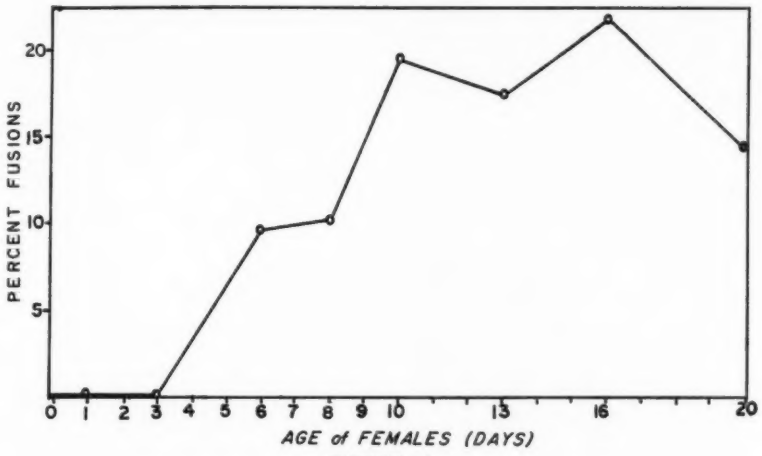


FIGURE 14
The frequency of chamber fusion as a function of age.

normal, although somewhat shrunken. Sugar-fed, *fused* flies show similar abnormalities (Figures 20-23). Developing tumors are filled with pycnotic nuclei. Nuclei which have undergone differentiation into nurse nuclei appear normal. Thus only undifferentiated, actively-dividing tissues degenerate under these circumstances. It appears that some nutrient essential for the maintenance of germarial and tumorous tissue is deficient in the diet of sugar-fed females. Thus the effects of the deficient medium include modification of both the germarial morphology and the stage distribution of oocytes. Whether or not the nutrient required for germarial maintenance is different from that required for further development of stage 6 oocytes is unknown.

According to Oster (1955) females which are fed on sugar-agar

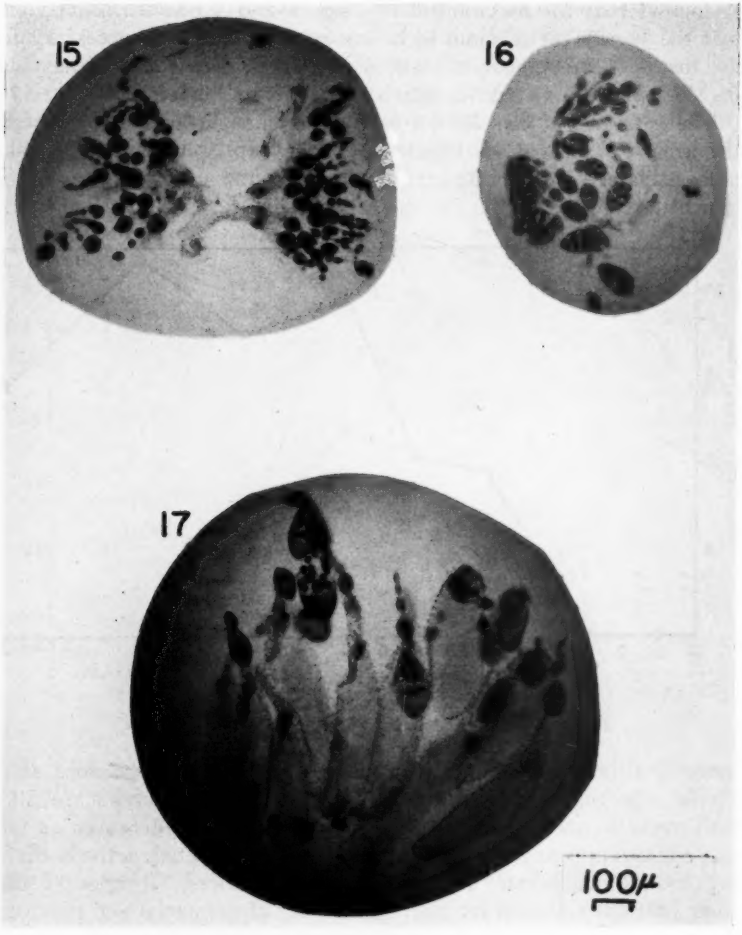
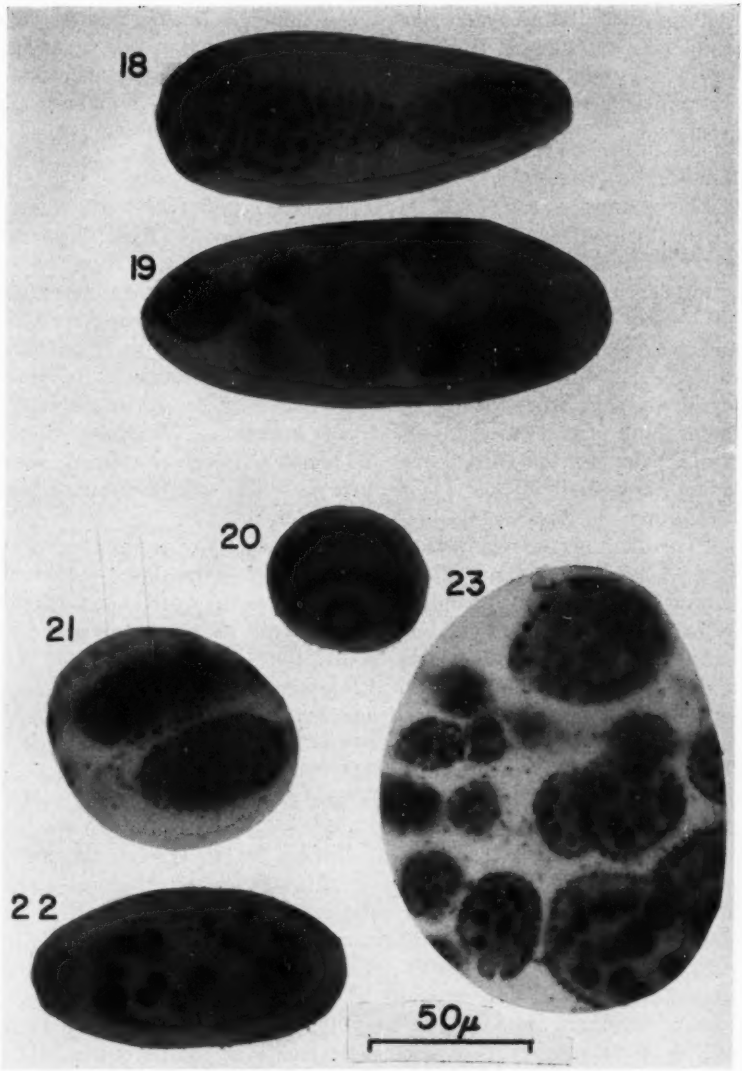


FIGURE 15
The ovaries of a 13 day *fu*/M5 female fed on sugar-agar.

FIGURE 16
One ovary of a freshly hatched *fu*/M5 female.

FIGURE 17
One ovary of a 13 day *fu*/M5 female fed on live yeast.



FIGURES 18 AND 19

The terminal portion of the ovaricle of a 13 day *fu*/M5 female fed on sugar-agar (19). Note that the germaria are spherical rather than sausage-shaped and that they contain little cellular detail. Compare with the normal ovariole (18).

FIGURES 20-23

Chambers from the ovaries of sugar-fed 8 day *fu* females. The chambers shown in 20 and 21 are tumorous. Note that the majority nuclei are pycnotic. The chamber shown in Figure 22 is partially tumorous. Note in 22 and 23 that the nurse nuclei appear relatively normal.

food for about 10 days have mostly oogonial cells in their ovaries. Our studies do not support this view. The ovaries of flies fed sugar-agar for 13 days contain at least 150 primary oocytes. Stages 2 through 6 are represented. Whether or not any viable oogonia remain in the germarium is unknown at present.

Studies of *female-sterile* and *narrow*^{2}

The mutants *female-sterile* (*fes*) and *narrow*^{2} (*nw*^{2}) are characterized by ovaries which are completely tumorous (Figures 24 and 25). With respect to external morphology *fes* is wild type in appearance. The spermathecae, accessory glands and ventral receptacle are present. Homozygous *nw*^{2} females also have all sperm receptacles and accessory glands. This mutant is characterized by its pointed wings. According to Waddington (1941) the difference in the shape of the *nw* wing from wild type is due to the differences in the rate of the division of the wing cells in varying directions. *Nw*^{2} and *fes* have a much longer longevity than *fu* females. Feulgen-stained whole mounts were made of *nw*^{2} and *fes* ovaries from females of known ages (5 through 30 days).

Depending on the age of the female the ovaries of *fes* and *nw*^{2} have 4 to 50 times the volume of the combined germarial tissue found in a normal ovary. It is obvious, therefore, that in both cases ovarian growth takes place, although no normal 16-cell chambers are produced. During the period studied (days 5-30) the *fes* ovary undergoes a 4-fold increase in size and ends up with a volume of 9×10^6 cubic micra. However, at best the *fes* ovary has a volume only 5% that of the mature wild type ovary. The ovaries of *fes* females are always larger than *nw*^{2} ovaries of the same age. During days 5 to 30 the *nw*^{2} ovary increases in volume from 0.8×10^6 to 5.2×10^6 cubic micra.

The ovarioles of *fes* females are subdivided into a series of sausage-shaped masses of cells, and each cell mass is surrounded by a rather ill-defined follicular epithelium (see also Gloor and Hadorn, 1942). These large follicles are often subdivided by transverse follicle cell walls into a series of rather ill-defined cysts. The follicles are filled with thousands of oogonia-like cells and hundreds of cells in various stages of pycnotic degeneration. A few cells in each chamber are found in mitosis. Usually the karyotype is normal, but some highly polyploid metaphases have been observed which contain a dozen or so pairs of rod-shaped chromosomes plus irregular blobs of Feulgen-

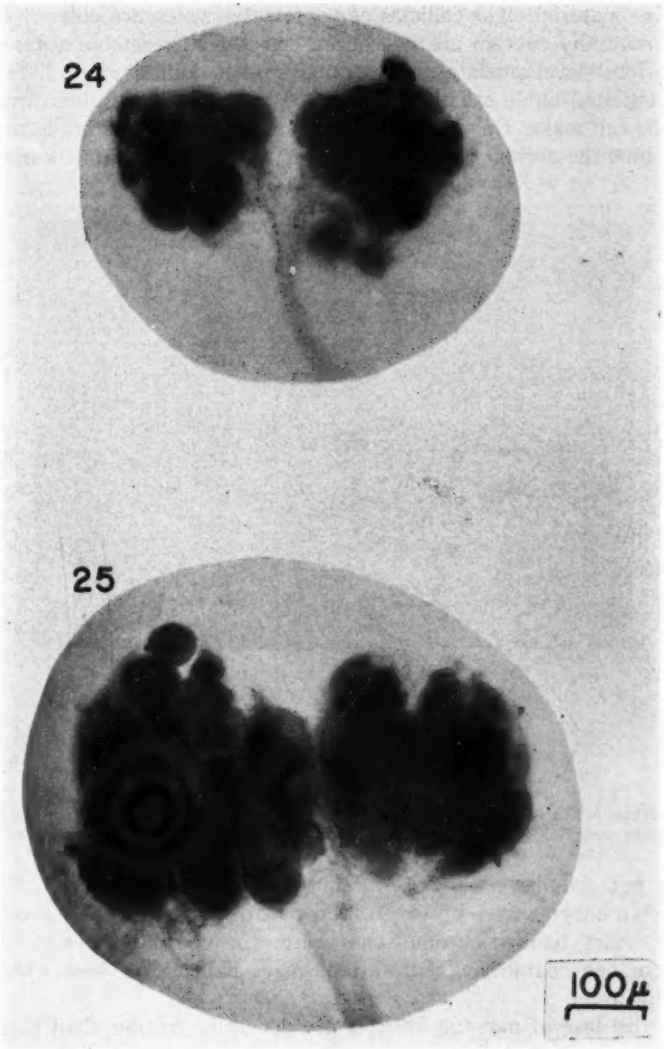


FIGURE 24
The ovaries of a yeast-fed *narrow*^{2} female (7 days old).
FIGURE 25
The ovaries of a yeast-fed *female-sterile* female (7 days old).

positive material. The follicles of *fes* females which are older than 10 days normally contain about a dozen cells which resemble nurse cells at various developmental stages up to and including stage 6. These differentiated nurse cells are distributed in a random fashion throughout the cell mass. One instance was observed where differentiation approaching the normal type occurred in a *fes* ovary. Four fairly normal,

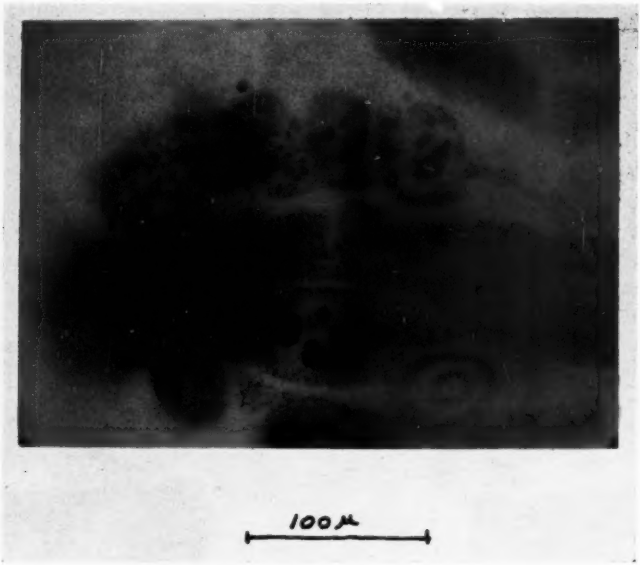


FIGURE 26
A portion of the ovary of a 13 day *fes* female. Note the 4 chambers which are nearly normal in morphology. One chamber contains thick, short, banded chromosomes.

early egg chambers were produced by a 13 day old female (Figure 26). No oocytes were observed in any of the chambers, however, and thick, short, banded chromosomes were observed in the nuclei of the cells of one chamber. Such chromosomes have never been observed previously.

In the case of nw^2 the follicles are generally smaller than those of *fes* females, and they are more often spherical than sausage-shaped. A well defined layer of follicle cells surrounds the tumorous mass. The

follicles are not subdivided into cysts. The karyotype of the metaphases that can be analyzed appears normal. The follicles often contain a dozen so differentiated nurse cells which are never more advanced than stage 4. As in the case of *fes* no oocytes have been observed (see also Beatty, 1949), pycnotic cells are very common, and most cells in the tumors resemble oogonia. Thus oocytes are rare or absent in *fes* and nw^2 tumors, but they are found in the *fu* tumors and in the radiation-induced ovarian tumors (King, 1957b).

According to Clancy and Beadle (1937) *fes* ovaries implanted into *fes* + hosts remain rudimentary. Sobels (1950) has shown that fes^+;*ltr* ovaries develop normally when implanted into *fes*;ltr^+ hosts.

Discussion

The three female-sterile mutations studied differ with respect to their degree of pleiotropism. The gene *fes* affects the ovaries, nw^2 affects both the ovaries and the wings, while *fu* affects the wings, ocelli, scutellum, certain bristles, the ovaries, and it lowers longevity and may terminate embryogenesis. However, it is assumed that each gene has a single primary action.

The germarium of *Drosophila melanogaster* may be divided into a series of four regions (Figure 27). The first region contains oogonia,

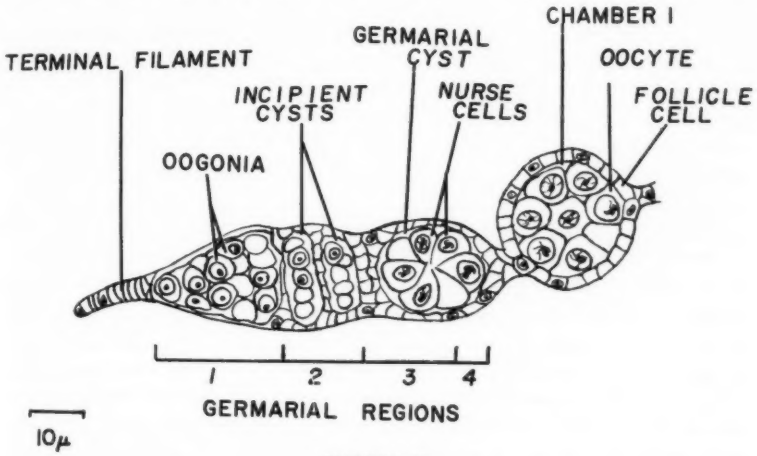


FIGURE 27

The apical portion of a *Drosophila* ovariole. Note the mesodermal cells lying between the oogonia and the incipient cysts.

the second contains incipient cysts, the third contains the nurse cells of the germarial cyst, and the fourth contains the oocyte. In region 1 cell division is unlimited; whereas in region 2 cell division is limited to 4 consecutive divisions (with the result that a 16-cell aggregate is formed). In region 3 further cell division ceases, but synthesis of DNA continues. The result is that nurse cell nuclei exhibit a range of DNA contents. The nuclei can be grouped into consecutive classes, each of which has ½ the DNA content of the succeeding class (Schultz, 1956). In region 4 such continued DNA synthesis is inhibited.

The *fes* females, *nw*^{2} females and old *fu* females are characterized by the fact that a restriction is not placed upon cell division. We conclude therefore that there is something abnormal about the germ cells of region 2 in the germaria of *fes*, *nw*^{2} and old *fu* females. Limited differentiation of oogonia into nurse cells does occur in *nw*^{2} ovaries and to a greater extent in *fes* ovaries. Nurse cell differentiation probably occurs in *fu* also, but here the situation is complicated because fusions of normal and tumorous chambers obscure the picture.

To explain the above series of facts we postulate that an inhibitory substance (I) is deposited near region 2 of the germarium by mesodermal cells lying basal to the oogonia. This substance interacts with the genetic machinery of germ cells entering region 2 so that a limitation is placed upon further cytokinesis. If the genetic machinery of these germ cells is damaged (by ionizing radiation, for example) this interaction may not occur even though I is present (cf. King, 1957b for further development of this idea). The elaboration of I is under gene control according to this theory, and the + alleles of *fes*, *nw*^{2} and *fu* are involved directly or indirectly in the synthesis of I. The *fes* + substance enters into a relatively restricted series of reactions. The results of the transplantation experiments of Clancy and Beadle and of Sobels indicate that the *fes* + substance is nondiffusible. The *nw* + substance is involved somehow in wing development as well as ovarian development. The *fu* + substance, however, is supposedly essential for many diverse processes. It is required for normal embryonic development, for the production of normal wings, ocelli, ocellar bristles, ovaries, and for normal adult longevity. We further postulate that the *fu* + substance is incorporated into the yolk of eggs produced by *fu*/+ females. However, the *fes* + substance and the *nw* + substance (the primary products of the + alleles of *fes* and *nw*) are not incorporated (or at least not in large enough amounts

to produce an effect) into the eggs produced by fes/+ and nw/+ females. Half of the eggs laid by *fu*/+ females will cast off the *fu* + allele in the polar bodies. If such eggs are fertilized by Y or *fu* sperm, a hemizygous or homozygous individual will result which can pass through embryogenesis normally because of the store of *fu* + substance in the yolk. We assume that the *fu* + substance also serves as a stable, non-diffusible precursor for compound I, that the *fu* + substance is incorporated into the anlage of the gonads, and therefore that the mesodermal cells bordering the oogonia in region 1 (of the germaria of the young *fused* females which develop) can produce I. However, the reserve of the *fu* + substance is used up as oocytes are produced, and it cannot be replaced. Tumorous chambers begin to develop once the concentration of I falls below some critical amount. This hypothesis would explain why as the fly ages the frequency of tumors increases. We have mentioned that instances are found in ovarioles where a normal chamber has tumorous chambers in front of and behind it. If we wish to keep the above hypothesis, we must modify it to include the concept that as I is used up it becomes non-homogeneously distributed. Cells entering a deficient area are not modified and and thus become tumorous, whereas those entering an area where the concentration of I remains high have a limitation placed upon their further division and thus develop into normal chambers.

Clancy and Beadle (1937) transplanted ovaries from *w sn*$^{+}$ *fu*, late third instar larvae into female *w*^{e}*sn fu*$^{+}$, host larvae of the same age. The eggs laid by those hosts which reached the adult stage were examined. The eggs produced by the implanted ovary could be distinguished from those produced by the host's ovary, since *sn* eggs have a characteristically abnormal short, blunt shape which is strikingly different from the normal appearance of the *fu* egg. A few host females did lay eggs which looked normal, and these females when mated to *fu* males produced no offspring. It would be premature to conclude from these results that the tissues of the host had no influence upon the developing implant and vice versa, since no cytological study was made of the ovaries of either host or donor.

Since *fu* females have abnormal wings; missing ocelli, ocellar bristles, scutellar bristles; few ovarioles; lowered longevity; etc., it is assumed that the fu + substance in the yolk does not enter into the reactions which are involved in the production of the above characteristics.

Summary

Descriptions are given of ovarian tumors produced by the X-chromosomal gene *fused* and the second chromosomal genes *female-sterile* and *narrow*.[2] The *fes* and *nw*[2] females have 100% tumors; whereas the frequency of tumors increases from 0-70% as *fu* females age. The feeding of flies on a deficient medium (sugar agar) results in modification of the germarial morphology and the stage distribution of oocytes. A mechanism is postulated to explain the induction of tumors in the ovaries of the mutant flies.

Acknowledgments

Data on fu^{ff} and fu^{57a} were collected by James E. Powell.

References

Beadle, G. W. 1931. A gene in maize for supernumerary cell divisions following meiosis. *Cornell Agric. Expt. Sta. Mem.*, **135**, 3-12.

Beadle, G. W. 1932. A gene in *Zea mays* for the failure of cytokinesis during meiosis. Cytologia **3**, 142-155.

Beatty, R. A. 1949. Studies on reproduction in wild-type and female sterile mutants of *Drosophila melanogaster*. *Proc. Roy. Soc. Edinburgh*, **63**, 249-270.

Clancy, C. W., & Beadle, G. W. 1937. Ovary transplants in *Drosophila melanogaster*. Studies of the characters *signed*, *fused*, and *female-sterile*. *Biol. Bull.*, **72**, 47-56.

Counce, S. J. 1956. Studies on female-sterility genes in *Drosophila melanogaster*. II. The effects of the gene *fused* on embryonic development. *ZIAV*, **87**, 462-481.

Crew, F. A. E., & Koller, P. 1936. Genetical and cytological studies on the intergeneric hybrid of *Cairina moschata* and *Anas platyrhynca platyrhynca*. *Proc. Roy. Soc. Edinburgh*, **56**, 210-241.

Gloor, H., & Hadorn, E. 1942. Vergleich der Schädigungen im Ovar von *Drosophila melanogaster*, bewirkt durch einen Letalfaktor und ein Sterilitätsgen. *Archiv der Julius Klaus Stiftung fur Vererbung—forschung, Sozialanthropologie und Rassenhygiene*, **17**, 438-440.

Gordon, M. 1953. The genetics of fish diseases. *Trans. Amer. Fish. Soc.*, **83**, 229-240.

Gruneberg, H. 1952. The genetics of the mouse. 2nd edition. M. Nijhoff. The Hague.

Huxley, J. 1956. Cancer Biology 1. Comparative and genetic. *Biol. Rev.*, **31**, 489-496.

———. 1957. Cancer Biology 2. Viral and epigenetic. *Biol. Rev.*, **32**, 1-37.

Dobzhansky, T. 1934. Studies on hybrid sterility. I. Spermatogenesis in pure and hybrid *Drosophila pseudoobscura*. *Zeits. Zellforsch.*, **21**, 169-223.

Kehr, A. E., & Smith, H. H. 1954. Genetic tumors in *Nicotiana* hybrids. Brookhaven Symposia in Biology: No. 6. Pp. 55-78.

King, R. C. 1955. A method for studying successive batches of eggs laid by treated or control *Drosophila*. *Am. Nat.*, **89**, 369-370.

———. 1957a. The cytology of the irradiated ovary of *Drosophila melanogaster*. *Exptl. Cell Res.*, **13**, 545-552.

———. 1957b. Oogenesis in adult *Drosophila melanogaster*. III. The radiation-induced ovarian tumors. *Growth*, **21**, 129-135.

———, & BURNETT, R. C. 1957. Hereditary ovarian tumors in *Drosophila melanogaster*. *Science*, **126**, 562.

———, & WOOD, E. M. 1955. Sex-linked lethal mutations induced by thermal neutrons in male and female *Drosophila melanogaster*. *Genetics*, **40**, 494.

LITTLE, C. C. 1939. Hybridization and tumor formation in mice. *PNAS*, **25**, 452-455.

———. 1947. The genetics of cancer in mice. *Biol. Rev.*, **22**, 315-343.

LYNCH, C. 1919. An analysis of certain cases of intra-specific sterility. *Genetics*, **4**, 501-533.

MULLER, H. J. 1954. The nature of the genetic effects produced by radiation. In A. Hollaender ed. Radiation Biology Vol. 1, Part 1, pp. 421-424. McGraw-Hill.

OSTER, I. I. 1955. A technique for treating male and female germ cells with chemical mutagens. *Drosophila Information Service*, **29**, 153-154.

PUCK, T. T., & FISHER, H. W. 1956. Genetics of somatic mammalian cells. I. Demonstration of the existence of mutants with different growth requirements in a human cancer cell strain (HeLa). *J. Exptl. Medicine*, **104**, 427-434.

REED, S. C. 1955. Counseling in Medical Genetics. W. B. Saunders.

RIZKI, M. T. M. 1957. Tumor formation in relation to metamorphosis in *Drosophila melanogaster*. *J. Morph*, **100**, 459-472.

ROTHENBUHLER, W. C., & J. W. GOWEN. 1955. Chromosomal localization of the hereditary basis for gyandromorph production in honeybees (*APIS Mellifera* L.). *Genetics*, **40**, 593-594.

SATINA, S., RAPPAPORT, J., & BLAKESLEE, A. F. 1951. Ovular tumors connected with incompatible crosses in *Datura*. *A. J. Botany*, **37**, 576-586.

SCHULTZ, J. 1956. The relatien of heterochromatic chromosome regions to the nucleic acids of the cell. *Cold Spring Harbor Symp. Quant. Biol.*, **21**, 307-328.

SOBELS, F. H. 1950. Experimentell erzeugte Nachkommenschaft von letalen ovarien der mutante *letal-translucida* (ltr) von *Drosophila melanogaster*. *Experientia* **6**, 139-140.

WADDINGTON, C. H. 1941. The genetic control of wing development in *Drosophila*. *J. Genetics* **41**, 75-139.

WHITING, P. W. 1943. Androgenesis in the parasitic wasp *Habrobracon*. *J. Heredity* **34**, 355-366.

WILSON, L. P., KING, R. C., & LOWRY, J. L. 1955. Studies on the tu^w strain of *Drosophila melanogaster*. I. Phenotypic and genotypic characterization. *Growth*, **19**, 215-244.

Growth, 1957, **21**, 263-280.

OOGENESIS IN ADULT *DROSOPHILA MELANOGASTER*. V. MUTATIONS WHICH AFFECT NURSE CELL NUCLEI[1]

Northwestern University, Evanston, Illinois and Brookhaven National Laboratory, Upton, N. Y.

R. C. KING AND R. G. BURNETT

(Received October 7, 1957)

INTRODUCTION

THERE are many instances where genes have been implicated in the control of various cytological processes. Among such genes are mutants like those studied by Beadle (1930) and Rhoades (1947) in maize, by Gowen (1933) and Fahmy (1952) in different species of *Drosophila*, by Bergner et al. (1934) in *Datura*, by Richardson (1935) in *Crepis* and by Li et al. (1945) in wheat which cause asynapsis or desynapsis.[2] Other mutants such as those studied by Beadle (1932) in maize and Bull (1956) in *Drosophila melanogaster* produce chromosomal stickiness. Mutant genes which cause abnormalities in spindle formation and cytokinesis have been described in different *Drosophila* species by Sturtevant (1929), Wald (1936), Lewis and Gengarella (1952), and Counce (1956) and in maize by Clark (1940). Still other genes which produce polyploidy have been described in barley by Smith (1942) and in *Drosophila hydei* by Staiger and Gloor (1952). Finally mutant genes are known which cause gynandromorphism (Goldschmidt and Katsuki, 1928, and Goldschmidt, 1957), somatic crossing over (Stern, 1936), and abnormal nuclear segmentation (Nachtsheim, 1950).

It is the purpose of this paper to describe oogenesis in sterile, female *Drosophila melanogaster* homozygous for any of the following eight genes: *female-sterile 2.1, singed*^{36a}, *raspberry*^{1}, *rotund, tiny, apterous*^{1}, *diminutive*, and *minus*. It will be shown that the plus alleles of these genes are implicated in the control of a specific group of cytological processes which involve the multiplication, differentiation and functioning of the chromosomes of the nurse cells of the developing eggs.

[1] Research supported by the U. S. Atomic Energy Commission (Contract No. AT (11-1), Project 12), the National Science Foundation (research grant NSF-G4816) and by the Graduate School of Northwestern University.

[2] See also c(1)a, eq and mot-36 e in Bridges and Brehme (1944).

Materials and Methods

Information as to the history of the mutants, the stocks used and their source, the age of the females providing the ovaries studied, etc. is presented in Table 1. All females were cultured with males. The methods used are identical to those described in an earlier paper (King, Burnett and Staley, 1957).

Results and Conclusions

Mutants Affecting the Replication of Nurse Cell Chromosomes

The normal behavior of nurse cell chromosomes has been described previously (King, Rubinson and Smith, 1956). The nurse cell chromosomes of *Calliphora erythrocephala* behave in a somewhat similar fashion (Bier, 1957).

(1) *female-sterile 2.1*

The ovaries of flies homozygous for this mutant gene contain germaria and oocytes in stages 1-4 which generally appear normal. Stages 5-9 are distinctly abnormal, however, with respect to the morphology of the nurse cell nuclei. The extent to which the synthesis of Feulgen-positive material occurs subsequent to stage 4 is markedly reduced with the result that nurse cell nuclei (particularly those of stages 6-8) appear relatively devoid of stainable material (Figure 1).

The nuclei of stage 3 nurse cells contain one short and five long chromosome arms. Presumably the five long arms represent the paired homologues of chromosomes X, 2 and 3, and the short arm is chromosome 4. These banded chromosomes appear to be attached to the nuclear membrane at their centromeric regions. Subsequent to stage 3 the chromosomes of the nurse cells of *fs 2.1* females follow either of two courses of development.

In one case the nurse nuclei continue to grow in volume until they reach a size characteristic of a stage 8 nucleus. Normally such nuclei would be filled completely with chromosomal material. Instead the five long chromosome arms are readily visible as fuzzy threads each about four micra in diameter and thirty micra long (Figure 2). The fuzzy appearance is due to Feulgen-positive chromonemal loops which originate from the longitudinal axis of the chromosomes. The diameter of the strands which form the loops is about 0.2 to 0.5 micra. After this stage the nuclei increase in volume and take on a pale, reticulate appearance. This morphological change presumably arises from the uncoiling of chromosomal gyres and the dispersing of the loose chromo-

TABLE 1
Information as to the mutants studied

Mutant	Abbreviation	Locus	Discovered by	Date	Stock	Source	Number of ovaries observed	Age (days) of adult ovaries
female-sterile 2.1	fs 2.1	2–57.6	M. Edmonson	1950	bur fs 2.1 / Cy $cn^2L^4sp^2$	Indiana Univ.	13	6
rotund	rn	2–54.5	H. B. Glass	1929	rn/SM1	California Inst. of Technology	7	5–8
singed^{36a}	sn^{36a}	1–21.0	W. P. Spencer	1936	sn^{36a}/M5	California Inst. of Technology	14	6
raspberry^4	ras^4	1–32.8	P. T. Ives	1938	ras^4m/ClB	Indiana Univ.	12	6
tiny	ty	1–44.5	C. B. Bridges	1925	g^2 ty/M5	California Inst. of Technology	14	6
apterous^4	ap^4	2–55.4	N. N. Medvedev	1932	ap^4/al^2Cy lt^vsp^2	California Inst. of Technology	12	1–3
diminutive	dm	1–4.6	E. Nichols-Skoog	1933	dm/M5	California Inst. of Technology	15	8
minus	mi	2–104.7	R. L. Biddle	1928	mi/Pm^2	California Inst. of Technology	8	8

somal strands throughout the nuclei. Not all nuclei in a given chamber make this change at the same time. The nurse nuclei of chambers equivalent in volume to a normal stage 10 reach their maximum size. Such nuclei contain a network of threads—each no more than 0.5 micra in diameter. Occasionally the most posterior nurse cell nucleus in a chamber may reach a volume of 1.5×10^5 cubic micra (two times the maximum volume normally observed).

The alternative path involves the normal transformation from stage 3 to stage 4. To do this the chromosomes shorten and thicken. The result is the production of a nucleus which contains one small and five large Feulgen-positive blobs which are connected to each other by fine strands. After this normal stage 4 the nurse nuclei increase in volume and gradually take on a pale reticulate appearance as in the first case. In both cases pale, reticulate nuclei first appear in chambers equivalent in volume to normal, late stage 9 chambers. Therefore, chambers equivalent in volumes to stages 5-9 show nurse cells with an abnormal nuclear morphology. The nuclei appear retarded since they contain chromosomes which are similar in morphology (although not in size) to those of stages 3 or 4.

Border cells are present and the follicular epithelium differentiates characteristically. Yolk synthesis appears to continue normally during stages 8-13. A normal chorion and micropyle are produced, and the oocyte nucleus is eventually converted into a karyosphere. The mature ovarian oocyte, however, may be abnormal in shape, and the dorsal appendages are often improperly formed because the posterior follicular epithelium breaks down prematurely. Apparently normal stage 14 oocytes have been observed, however. Degeneration of *fs 2.1* oocytes often occurs prior to stage 13. Fusions of adjacent chambers occur rarely.

Normally the Feulgen-positive material of the nuclei of the nurse cells undergoes a limited series of doublings (Freed and Schultz, 1956, Schultz, 1956). We interpret the above observations to indicate that the number of doublings of DNA is reduced in the *fs 2.1* ovary. The effect appears to be produced prior to stage 5, and as a result there is probably an abnormally low DNA concentration in the nuclei of nurse cells in stages subsequent to and including stage 5.

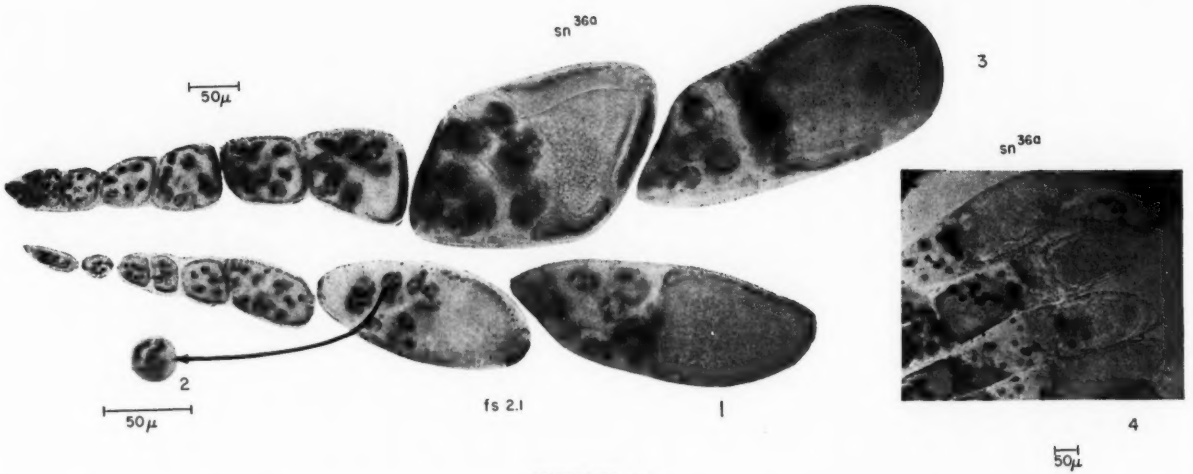
The abnormal chromosomal morphology also may result from an abnormal coiling cycle, such as has been described in various plants (Lesley and Frost, 1927; Upcott, 1937, and Rhoades, 1955).

(2) *singed*^{36a}

Females homozygous for *sn*^{36a} have gnarled bristles. The ovaries of

such flies show germaria and oocytes in stages 1 through 4 which appear relatively normal. The mutant gene therefore appears to produce its effect somewhat later in development than *fs 2.1*. Chambers equivalent in volumes to stages 5-9 show nurse cells with an abnormal nuclear morphology. The nuclei appear retarded (Figure 3), since they contain chromosomes which are similar in morphology (although not in size) to those of late stage 4 chambers. Thus the sn^{36a} ovary appears to develop normally until the nurse cell chromosomes pass the compact stage characteristic of stage 4. The chromosomes next begin to uncoil and so form tangled bulbous structures that are less densely strained. Large plasmosomes are produced, and the nurse nuclei increase markedly in volume. Chambers equivalent in volumes to stages 6 and 7 have nuclei with volumes characteristic of stages 8-10. Chambers corresponding in volumes to stages 3-7 are abnormal in shape (they are often wider than they are long), which may indicate that the passage of oocytes down the ovariole is impeded, and adjacent eggs are therefore pressed together. As yolk formation commences the nurse cell nuclei take on their characteristic pale, reticulate form. Border cells make their usual migrations. Once stage 11 is reached the nurse cell nuclei degenerate and disappear (Figure 4). The egg laid is generally a modified stage 11 oocyte. It contains only about 2/3 the normal quantity of yolk and has a clear anterior area which represents the nurse chamber. A partially formed chorion is present. Rarely more mature eggs with abnormally shaped filaments are produced (cf. Mohr, 1922). Fusions of adjacent chambers were not observed in sn^{36a}, although they are common in the ovaries of flies homozygous for other sterile *sn* alleles. The ovary of the sn^{36a} female therefore may be characterized by the abnormal behavior of the endopolyploid nurse cell nuclei late in stage 4. The production of further quantities of Feulgen-positive material is inhibited and an excessive synthesis of plasmosomal material occurs. The endopolyploid bristle and socket cells behave abnormally in sn^{3} pupae (Lees and Waddington, 1942).

Forty ovaries of $singed^{36a}$ *fused* females varying in age from 6-16 days were examined and were found to show abnormalities characteristic of both mutants. This indicates that the genes affect independent processes. As in *fu* tumors and fusions (Figure 5) between adjacent developing eggs occur (cf. King, Burnett and Staley, 1957). However, the tumor frequency is reduced below that of *fu* flies of the same age. Most non-tumorous eggs are able to develop farther than is characteristic for sn^{36a}. Numerous pycnotic, stage 12



FIGURES 1-4

(1) An ovariole from an *fs 2.1* female. The germarium and seven developing oocytes are shown. The nuclei of the nurse cells in chambers 3-6 appear abnormal, since they are relatively devoid of Feulgen-positive material. (2) An enlarged view of lampbrush chromosomes in one of the nurse cell nuclei from the 6th chamber in Figure 1. (3) An ovariole from a *sn^{36a}* female. The germarium and nine developing oocytes are shown. Note that the abnormally large nurse cell nuclei are relatively devoid of Feulgen-positive material and contain giant plasmosomes. Note also the relatively squat appearance of the chambers. (4) A group of four stage 11 oocytes lying parallel to each other. Most eggs of *sn^{36a}* females are laid at this stage. Note the degenerating nurse cell nuclei in anterior portion of each egg. Each egg contains only about 2/3rds the normal quantity of yolk.

FIGURE 5

An ovarian preparation from an 8 day old *sn*^{36a} *fu* female. Note the four stage 14 oocytes which have fused together to form a chain.

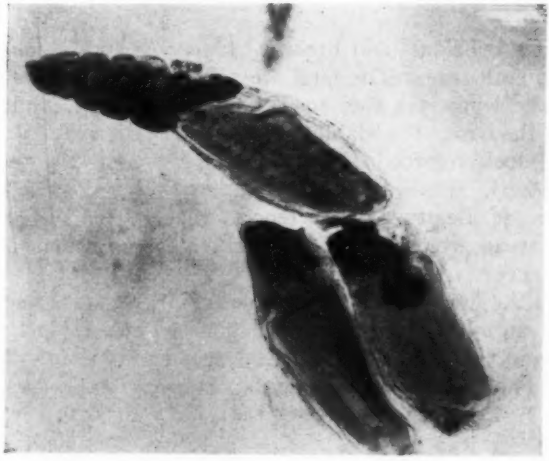


FIGURE 6

Another ovarian preparation from an 8 day *sn*^{36a} *fu* female. Note the Feulgen-positive material lying in the right-most oocyte. This material originates from degenerating nurse cell nuclei.

and 13 oocytes are observed, as well as an occasional stage 14 with abnormally short filaments. Degenerating nurse cell nuclei are often observed inside the oocyte (Figure 6). The characteristic retardation in the synthesis of Feulgen-positive material in nurse cell nuclei is absent. It should be pointed out, however, that we do not have at our disposal coisogenic strains of *fu*, sn^{16a}, and *fu* sn^{16a}; so that a different modifier system may be operating in each stock.

(3) *raspberry*^{1}

Females homozygous for *raspberry*^{1} have a ruby eye color. Oogenesis in the *ras*^{1} ovary resembles that in sn^{16a} ovaries as far as pre-yolk stages are concerned and that in *fs 2.1* as far as post-yolk stages are concerned. That is, developing eggs contain abnormal nurse cell nuclei (Figure 7) as in the case of sn^{16a}, but some eggs are able to continue past stage 11. One or two stage 14 oocytes are seen in each ovary. These mature ovarian oocytes are abnormal in shape, however. They are short and squat as in the case of *fs 2.1*. Fusions of adjacent egg chambers occur commonly.

(4) *rotund*

According to Bridges and Brehme (1944) the gene *rotund* produces sterility in both sexes. The tarsi are three-jointed and sex combs are absent. The wings are shortened, but are normal in width and are warped at the base. The viability of *rotund* flies is reduced. The nurse cell nuclei look retarded (Figure 8), and the picture seen is very similar to *ras*^{1}. However, many apparently normal stage 14 oocytes are produced. Beatty (1949) reports that *rn* females occasionally produce larvae which die at an early stage. Fusions of adjacent chambers occur.

Thus *fs 2.1*, sn^{16a}, *ras*^{1}, and *rn* ovaries have a characteristic abnormality, an inhibition in the production of Feulgen-positive material in the nurse cell nuclei and an excessive synthesis of plasmosomal material. These abnormalities may be observed readily by contrasting Figures 1, 3, 7 and 8 to Figure 9.

Mutants Affecting Yolk Synthesis

(1) *apterous*^{1}

Females homozygous for *ap*^{1} have their wings and halteres reduced to traces. Such females are late to eclose, manage to live for only 3 or 4 days, and are sterile. According to Beatty (1949) the accessory glands are small or missing and the spermathecal capsules are flat-

FIGURES 7, 8, 9

(7) An ovarian preparation from a 6 day old *ras*^{1}*m* female. Note the abnormal appearance of the nurse cell nuclei. (8) An ovarian preparation from a 5 day old *rn* female. Note the abnormal appearance of the nurse cell nuclei. (9) An ovarian preparation from a 4 day old, wild type female (Oregon R). Note that the nurse cell nuclei are filled with Feulgen positive material.

tened. Males homozygous for ap^4 are also sterile (Bridges and Brehme, 1944). Oogenesis proceeds normally in the ap^4 ovary until stage 7 is completed. The nurse cells next undergo pycnotic degeneration and the developing egg breaks down (Figure 10). Yolk formation never gets underway.

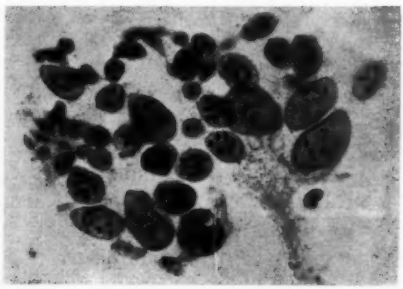


FIGURE 10

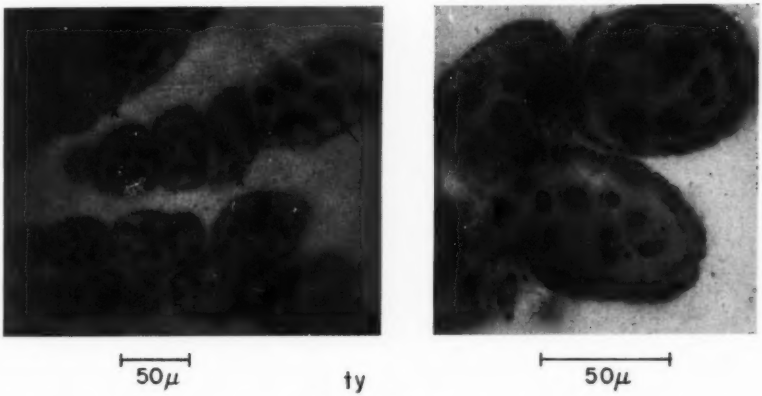
An ovarian preparation from a 3 day old ap^4 female. Note that only oocytes in preyolk stages are present.

Adult ap^4 females were fed upon medium to which pyronin (Gurr) was added. One hour later the brilliant red digestive system could be seen readily through the abdominal wall of the flies. Upon dissection the lumen of both the crop and the ventriculus was found to be filled with red food. The contents of the crop and the ventriculus were next examined and found to contain thousands of stained yeast cells. It is therefore obvious that ap^4 females do die prematurely and fail to synthesize yolk, but not because they are unable to ingest food.

(2) *tiny*

Females homozygous for *ty* have a small body covered with small bristles. Such females are late to eclose. Oocytes in stage 1 through 6 appear normal. The nucleoplasm of stage 7 nurse cells undergoes abnormal growth. There are produced as a result pale, reticulate nuclei (30 micra in diameter) which are similar in size and morphol-

ogy of those of stage 9 nurse cells (Figure 11). The nuclei next become pycnotic and degenerate. The cuboidal follicular epithelium becomes double layered in the region about the oocyte (Figure 12). Yolk formation does not occur to any significant degree. Most pycnotic cells have a volume of about 2×10^5 cubic micra and therefore correspond to stage 7. Pycnotic stage 4 and 6 chambers occur commonly. Fusions of adjacent chambers have not been observed among the 1400 developing eggs which were studied.



FIGURES 11-12

(11) Ovarioles from a *ty* female. No oocytes more advanced than stage 7 are present. The arrow points to a giant nurse cell nucleus. Xs mark degenerating chambers. (12) A *ty* chamber with pycnotic nurse cell nuclei and a double layered follicular epithelium.

It therefore is postulated that the + allele of *ty* controls yolk synthesis directly or indirectly. The proper differentiation of the follicular epithelium into a single layered sheet of squamous cells above the nurse cells and into a single layered sheet of columnar cells above the oocyte evidently must depend upon the proper synthesis of yolk.

(3) *diminutive*

Females homozygous for *dm* have a small, narrow body covered with small, slender bristles. Oocytes in stages 1 through 6 appear normal. The nucleoplasm of stage 7 nurse cells undergoes abnormal

growth. There are produced as a result pale-staining reticulate nuclei (15 micra in diameter) which are similar in size and morphology to those of stage 10 nurse cells (Figure 13). The chamber continues to grow until it reaches a volume of 1×10^6 cubic micra (equivalent to a stage 8), and it then degenerates. The enlarged nurse nuclei shrink and form densely-stained spheres. Degenerating stage 6 and 8 chambers also occur. Fusions of adjacent chambers occur rarely. Yolk is produced and the follicular epithelium differentiates into a single layer of squamous cells above the nurse cells. However, follicle

FIGURE 13
Degenerating *dm* chambers. Note the bizarre shapes of some of the nurse cell nuclei.

cells are generally cuboidal above the oocyte or they may become squamous here as well. The nuclei of the layer of follicular cells above the nurse cells may reach the condition normally seen in stage 9 where they lie as much as 20 micra apart. The oocytes of *dm* females are able to proceed farther in development than those of *ty* or *ap*^{1}, and some yolk is synthesized. However, in both cases abnormal nuclear growth is first noticed in stage 7 nurse cells and excessive nucleoplasmic material is synthesized.

(4) *minus*

As in the case of *ty* and *dm*, females homozygous for *mi* have a small body covered with small bristles. Such females are late to eclose. The *mi* ovary is similar to that of *dm* females. Some oocytes manage, however, to develop past stage 8; and apparently normal stage 10 and

14 oocytes are seen occasionally. Fusions of adjacent chambers occur very often (Figure 14).

Nonsterile Alleles of Female Sterile Mutants

It is interesting to note that females homozygous for the mutants *sn*^{3}, *sn*$^{3\delta c}$, or *ap*^{56f} (Burdick, 1956) (which are fertile alleles or pseudoalleles of female sterile mutants) have reproductive systems which

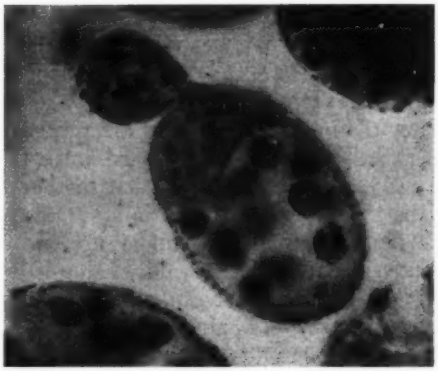


FIGURE 14

An ovarian preparation from an 8 day old *mi* female showing a compound chamber which resulted from the fusion of two adjacent chambers.

appear cytologically normal. The ap^{4}/ap^{56f} females are fertile. Elevated frequencies of pycnotic, stage 8 oocytes are observed, but otherwise the ovaries appear normal.

Discussion

The + alleles of the mutant genes *fs 2.1*, *sn*^{3a}, *ras*^{4} and *rn* appear to affect chromosomal replication in a precise fashion in a specific tissue (the nurse cells of the developing egg) at a specific time in the life cycle, but the effects of the genes are obviously different, since the morphological abnormalities they produce are easily distinguished.

Abnormalities in nuclear behavior occur earliest in the *fs 2.1* ovary. In *fs 2.1*, sn^{36a}, ras^{4} or *rn* ovaries many oocytes with abnormal nurse cell nuclei continue to grow and differentiate fairly normally. Contrary to expectations it is the sn^{36a} oocyte which ceases its development at the earliest stage. However, even the handicapped sn^{36a} oocyte has managed to pass from stage 5 through stage 11 (this developmental period normally requires 1 day). The genes sn^{36a}, ras^{4} and *rn* produce other obvious phenotypic effects in addition to female sterility.

Replication of nurse cell chromatin occurs in an apparently normal fashion in ap^{4}, *ty*, *dm* and *mi* oocytes. The plus alleles of these three genes presumably act later in oogenesis than do those of *fs 2.1*, sn^{36a}, ras^{4} and *rn*. With the exception of the *mi* ovary, oogenesis comes to a halt shortly after the first abnormalities are recognized. Oocytes of *dm* females can proceed to a more advanced stage than can ap^{4} and *ty* oocytes. Hsu (1952, 1953) has shown that the nurse cell nuclei are responsible for the synthesis of proteid yolk. It seems reasonable to assume that the plus alleles of ap^{4} and *ty* are involved directly or indirectly at some very early stage in yolk synthesis, whereas the plus allele of *dm* and *mi* come into action at some later step. Yolk formation in the *dm* oocyte comes to a halt prematurely at a stage where the ooplasmic volume is only 1/130th the maximum volume found in the mature ovarian primary oocyte. A few *mi* oocytes manage to complete development. The ty^{+}, dm^{+}, and mi^{+} genes are also similar in that they control the size of the bristles and over-all body size.

It is obvious that there is no direct parallelism between the normal synthesis of Feulgen-positive material in the nuclei of nurse cells and yolk deposition, since we have seen developing eggs with cytologically abnormal nurse nuclei undergo some yolk synthesis and developing eggs which are incapable of synthesizing yolk which have apparently normal amounts of DNA.

This paper is the fifth in a series devoted to the general topic of oogenesis in *Drosophila*. From the data presented in these papers there emerges a superficial description of the way in which a fruit fly goes about forming an egg. There are estimates as to the lengths of time required for the egg to go through various developmental stages and the growth rates involved. More important, it appears that various steps in the developmental sequence are controlled directly or indirectly by a group of at least eleven non-allelic genes. In Figure 15 the sequence of events in a *Drosophila* ovariole is presented in a

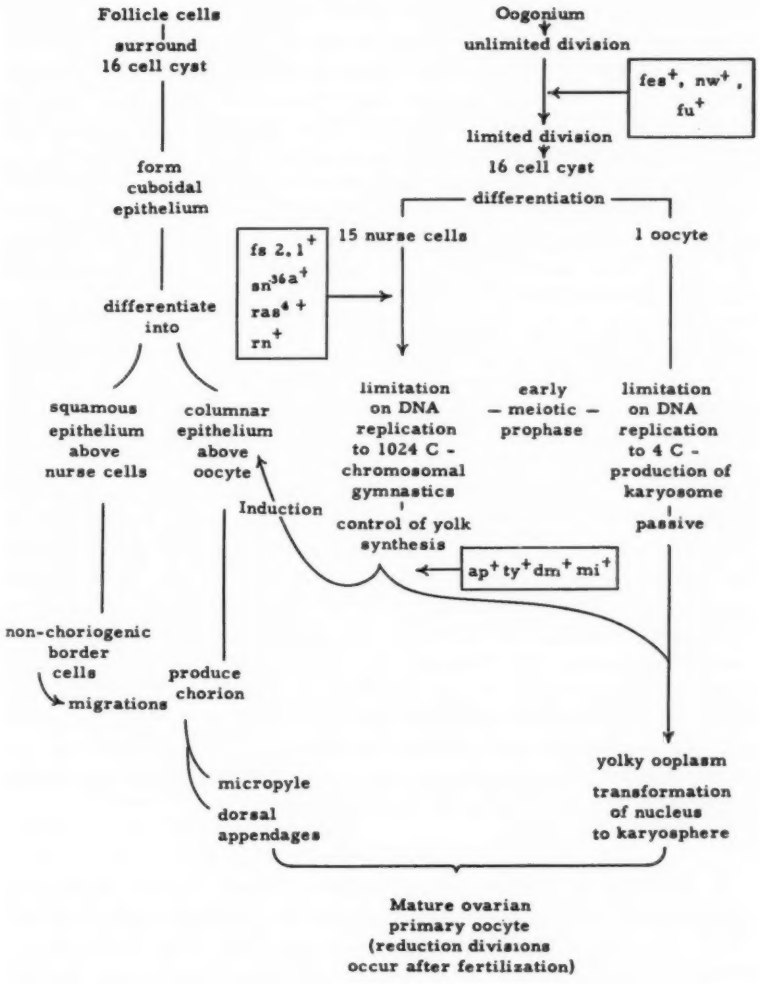


FIGURE 15

The sequence of morphogenetic events in a *Drosophila* ovariole. C represents the haploid amount of DNA. Follicle cells are mesodermal in origin. Oogonia originate from pole cells.

diagrammatic fashion. It is not known why the follicle cells take it upon themselves to surround the cyst of 16 germ cells; but once they do, their further differentiation seems to depend upon their location. Follicle cells which differentiate into choriogenic, columnar cells lie above yolky cytoplasm, and perhaps some agent in yolk induces this type of differentiation. Squamous, non-choriogenic, follicle cells are found above nurse cells, and it is from these non-choriogenic cells that the migrating border cells originate. There is evidence that at least three genes are involved in placing a limitation upon the division of oogonia in a specific region of the germarium, but the mechanism involved is unknown. It is not known specifically what causes 15 of the germ cells in the chamber to differentiate into nurse cells and the 16th cell to become the oocyte. The oocyte and the nurse cells behave very differently with respect to the synthesis of Feulgen-positive material. The genetic material of the nurse cells which have reached their maximum size may have undergone as many as nine consecutive doublings; whereas the genetic material of the oocyte has undergone but one such duplication. These differences in nuclear growth and differentiation are possibly reflections of the dominant role played by the nurse cell nuclei in vitellogenesis versus the passive role played by the oocyte nucleus. There is evidence that at least four genes control the replication and differentiation of the nurse cell chromosomes, but again the mechanism is obscure. The meaning of the various gymnastics the nurse cell chromosomes undergo, and what relationship these gymnastics have to yolk synthesis is also a mystery. Finally, it is known that at least four genes play an important role in vitellogenesis. The surface of this problem barely has been scratched, but the results are promising enough to warrant further work with the female sterile mutations of *Drosophila melanogaster*.

Summary

A description is given of oogenesis in sterile female *Drosophila melanogaster* homozygous for any one of the following genes: *female-sterile 2.1*, *singed*^{36a}, *raspberry*^{1}, *rotund*, *apterous*^{1}, *tiny*, *diminutive* or *minus*. The first four mutants apparently affect replication and differentiation of the nurse cell chromosomes. The second four mutants act later in oogenesis. They appear to disturb the proper functioning of the nurse cell nuclei during the synthesis of yolk.

References

BEADLE, G. W. 1930. Genetical and cytological studies of Mendelian asynapsis in *Zea mays*. *Cornell Univ. Agr. Exp. Sta. Memoir*, **129**, 1-23.

———. 1932. A gene for sticky chromosomes in *Zea mays*. *Z. indukt. Abst. Vererb.*, **63**, 195-217.

BEATTY, R. A. 1949. Studies on reproduction in wild-type and female-sterile mutants of *Drosophila melanogaster* (Meig.). *Proc. Roy. Soc. Edinb.*, **63B**, 249-270.

BERGNER, A. D., CARTLEDGE, J. L., & BLAKESLEE, A. F. 1934. Chromosome behavior due to a gene which prevents metaphase pairing in *Datura*. *Cytologia*, **6**, 19-37.

BIER, K. 1957. Endomitose und Polytänie in den Nährzellkernen von *Calliphora erythrocephala* Meigen. *Chromosoma*, **8**, 493-522.

BRIDGES, C. B., & BREHME, K. S. 1944. The mutants of *Drosophila melanogaster*. Carnegie Inst. of Washington Publ. 552.

BULL, A. L. 1956. Developmental effects of the vg^B deficiency in *Drosophila melanogaster*. *J. Exp. Zool.*, **132**, 467-507.

BURDICK, A. B. 1956. apterous^{56f}. *Drosophila Information Service*, **30**, 69.

CLARK, F. J. 1940. Cytogenetic studies of divergent meiotic spindle formation in *Zea mays*. *A. J. Bot.*, **27**, 547-559.

COUNCE, S. J. 1956. Studies on female-sterility genes in *Drosophila melanogaster*. I. The effect of the gene *deep orange* on embryonic development. *Z. indukt. Abst. Vererb.*, **87**, 443-461.

FAHMY, O. G. 1952. Maturation, fertilization and cleavage in normal eggs and in the presence of the cross-over suppressor gene. *J. Genetics*, **50**, 486-506.

FREED, J. J., & SCHULTZ, J. 1956. Effect of the Y chromosome on the DNA content of ovarian nuclei in *Drosophila melanogaster* females. *J. Histochem., & Cytochem.*, **4**, 441-2.

GOLDSCHMIDT, R. B. 1957. A remarkable action of the mutant "rudimentary" in *Drosophila melanogaster*. *PNAS* **43**, 731-36.

GOLDSCHMIDT, R. B., & KATSUKI, K. 1928. Zweite Mitterlung über erblicher Gynandromorphismus bei *Bombyx mori* L. *Biol. Zentralbl.*, **48**, 39-43.

GOWEN, J. W. 1933. Meiosis as a genetic character in *Drosophila melanogaster*. *J. Exp. Zool.*, **65**, 83-106.

HSU, W. S. 1952. The history of the cytoplasmic elements during vitellogenesis in *Drosophila melanogaster*. *Q. J. Micro. Sci.*, **93**, 191-206.

———. 1953. The origin of proteid yolk in *Drosophila melanogaster*. *Ibid.*, **94**, 23-8.

KING, R. C., BURNETT, R. G., & STALEY, N. A. 1957. Oogenesis in adult *Drosophila melanogaster*. IV. Hereditary ovarian tumors. GROWTH, **21**, 239-261.

KING, R. C., RUBINSON, A. C., & SMITH, R. F. 1956. Oogenesis in adult *Drosophila melanogaster*. GROWTH, **20**, 121-157.

LESLEY, M. M., & H. B. FROST. 1927. Mendelian inheritance of chromosome shape in *Matthiola*. *Genetics*, **12**, 449-460.

LEES, A. D., & C. H., WADDINGTON. 1942. The development of the bristles in normal and some mutant types of *Drosophila melanogaster*. *Proc. Roy. Soc.* London, **131B**, 87-110.

LEWIS, E. B., & GENGARELLA, W. 1952. *Claret* and non-disjunction in *Drosophila melanogaster*. *Rec. Gen. Soc. Amer.*, **21**, 44-45.

LI, H. W., PAO, W. K., & LI, C. H. 1945. Desynapsis in the common wheat. *A. J. Bot.*, **32**, 92-101.

MOHR, O. L. 1922. Cases of mimic mutations and secondary mutations in the X-chromosome of *Drosophila melanogaster*. *Z. indukt. Abst. Vererb.*, **28**, 1-22.

NACHTSHEIM, H. 1950. The Pelger-anomaly in man and rabbit. *J. Heredity,* **41**, 131-137.

RHOADES, M. M. 1947. Crossover chromosomes in unreduced gametes of asynaptic maize. *Genetics,* **32**, 101.

———. 1955. The cytogenetics of maize. In *Corn and Corn improvement.* G. F. Sprague, editor. Academic Press, pg. 124.

RICHARDSON, M. M. 1935. Meiosis in Crepis. II. Failure of pairing in *Crepis capillaris. Genetics,* **31**, 119-143.

SCHULTZ, J. 1956. The relation of the heterochromatic chromosome regions to the nucleic acids of the cell. *Cold Spring Harbor Symp. Quant. Biol.,* **21**, 307-328.

SMITH, L. 1942. Cytogenetics of a factor for multiploid sporocytes in barley. *A. J. Bot.,* **29**, 451-456.

STAIGER, H., & GLOOR, H. 1952. Mitose-hemmung und Polyploidie durch einen Letal-faktor (lpl = letal-polyploid) bei *Drosophila hydei. Chromosoma,* **5**, 221-245.

STERN, C. 1936. Somatic crossing over and segregation in *Drosophila melanogaster. Genetics,* **21**, 625-730.

STURTEVANT, A. H. 1929. The *claret* mutant type of *Drosophila simulans*: a study of chromosome elimination and of cell lineage. *Z. wiss. Zool.,* **135**, 323-55.

UPCOTT, M. 1937. Timing unbalance at meiosis in pollen sterile *Lathyrus odoratus. Cytologia,* Fujii Jubilee volume, pp. 299-310.

WALD, H. 1936. Cytologic studies on the abnormal development of the eggs of the *claret* mutant type of *Drosophila simulans. Genetics,* **21**, 264-81.

Growth, 1957, **21**, 281-285.

OOGENESIS IN ADULT *DROSOPHILA MELANOGASTER*. VI. A COMPARISON OF OOGENESIS AMONG *DROSOPHILA MELANOGASTER, VIRILIS, PSEUDOOBSCURA* AND *GIBBEROSA*[1]

R. C. KING AND MARILYN FLEISCHER WOLFSBERG[2]

Northwestern University, Evanston, Illinois, Brookhaven National Laboratory, Upton, N. Y. and New York University, N. Y., N. Y

(Received November 7, 1957)

INTRODUCTION

IT IS the purpose of this paper to compare oogenesis in four species of fruit flies. Three species with considerably different developmental times were chosen, *viz., Drosophila melanogaster, virilis* and *gibberosa*. In addition *D. pseudoobscura*,[3] which has a developmental time similar to that of *D. virilis*, was studied because of its rhythmical pattern of egg production (Donald and Lamy, 1936-1937).

MATERIALS AND METHODS

Table 1 presents data on the strains and source of each species investigated. The reader is referred to King, Rubinson and Smith (1956) for descriptions of the stage designations used in this paper. All flies were raised at 25° C on medium prepared according to the formula of King and Wood (1955). Egg laying behavior of mated females was determined according to the method of King (1955). Microscopic examination was made of ovarian whole mounts stained according to the Feulgen procedure. The weights of the eggs in mg were calculated by substituting the appropriate values for l (the length measured in microns) and w (the width measured in microns) in the equation $W = 0.5236 \times 10^{-9}\ w^2 l$. We assume a density of 1.

RESULTS AND CONCLUSIONS

The results of this study are summarized in Tables 1 and 2. The time required to reach sexual maturity is longer in those species re-

[1] Research supported by the U. S. Atomic Energy Commission.

[2] Graduate Student, Biology Department, New York University.

[3] A more detailed description of oogenesis and egg laying behavior in *D. pseudoobscura* will be given in a manuscript now in preparation.

quiring a relatively long time for pre-adult development. The average number of primary oocytes per mature adult female is not strikingly different among the various species. *D. virilis* seems to be able to produce the most eggs in proportion to its body size. However, it should be pointed out that both adult weight and daily egg production are highly variable characters. *D. gibberosa* lays so few eggs that we

TABLE 1

A comparison of 4 *Drosophila* species with respect to developmental times and egg production at 25° C.

species	*melanogaster*	*virilis*	*pseudoobscura*	*gibberosa*
strain	Oregon R	Japan	standard, Piñon Flat	Mexico
source	Harvard U.	Yale U.	Harvard U.	Columbia U.
avg. minimum time (days) from fertilization to eclosion	9	12	14	16
avg. minimum time (days) from eclosion to sexual maturity for female	3	4	4	8
avg. wt. (mg) of mature adult female	1.3	2.9	1.4	5.1
avg. calculated wt. (μg) of mature egg	9.5	11.1	6.3	20.3
avg. no. primary oocytes per ovariole	7.5	6.5	6.0	7.0
avg. no. ovarioles per ovary	12	17	18	17
avg. no. primary oocytes per mature adult female	180	220	216	240
avg. maximum no. of eggs laid daily per female	60	130	61	12
wt. of eggs laid daily/body weight	0.438	0.498	0.275	0.048

believe the fault lies in our being unable to provide this species with a medium suitable for its development.

At eclosion the four species differ markedly with respect to the condition of the ovary. The ovary of *D. melanogaster* is the most, that of *D. gibberosa* the least mature. Bucher (1957) has reported that in *D. melanogaster* the first follicle (stage 2 in our terminology) splits off from the germarium 60 hours after puparium formation. Thus, the ovary of a freshly eclosed *gibberosa* female resembles that of a *melanogaster* female which is three-quarters of the way through the pupal

stage. During the period of maximum oviposition the most posterior chamber in any ovariole generally belongs to stage 14 in *D. melanogaster*. In *virilis* stage 10 and 14 oocytes are equally abundant; this may indicate that stage 14 oocytes are kept in the *virilis* ovary a

TABLE 2

Ovarian maturity as a function of age for 4 species of *Drosophila*

Species	Number of ovaries observed	Age (days) of adult female	The most posterior chamber in an ovariole generally belongs to stage
melanogaster	13	0	7
	18	1	8
	10	2	9
	17	4	14*
	10	7	14*
	50	10	14*
	35	21	14*
	16	28	14*
	5	40	14*
	3	69	14*
virilis	18	0	4 or 5
	12	3	10
	16	7	10 or 14*
	10	10	10 or 14*
	12	17	10 or 14*
pseudoobscura	8	0	5
	11	1	6
	15	3	7, 10
	17	4	10, 14
	23	5	10, 12, 14*
	17	6	10*
	14	7	10, 14*
	17	8	7, 14*
	14	9	14*
	12	10	7, 14*
	17	11	14*
	13	12	14*
	3	13	14*
	5	14	14*
	15	21	14* (3 ovaries in stage 7)
	6	24	10 or 14*
	4	31	14*
	11	42	14*
	6	85	14*
gibberosa	18	0	2
	14	5	7 or 8
	6	6	8 or 10
	12	8	10
	8	9	10 or 14*
	8	12	14*

*ovaries from actively laying females

shorter time than in the *melanogaster* ovary. Sexually mature *D. pseudoobscura* females do not lay eggs relatively continuously the way the other species do but lay eggs for one or two days and then rest for one day. During periods of egg laying the most posterior chamber in each *pseudoobscura* ovariole is in either stage 10 or 14. During rest periods the most posterior oocyte is stage 7. It is known (Day, 1943) that extirpation of the ring gland in *Lucilia sericata* and *Sarcophaga securifera* prevents development of the egg beyond pre-yolk stages. The corpus allatum is also known to be necessary for the formation of mature eggs in *Rhodnius* (Wigglesworth 1936), *Drosophila* (Vogt 1943) and *Calliphora* (Thomsen 1952). To explain the above behavior we therefore suggest that in *pseudoobscura* a hormone responsible for the initiation of vitellogenesis (possibly elaborated by the corpus allatum) is secreted discontinuously. Whenever this hormone falls below a critical concentration immature oocytes cannot develop beyond stage 7. When the necessary threshold is reached, vitellogenesis proceeds (stages 8-12) until the mature egg (stage 14) is formed and laid.

Summary

A comparative study of oogenesis in four species of *Drosophila* has revealed striking differences with respect to the degree of maturity attained by the oocytes located in the most posterior chambers of the ovarioles of freshly eclosed and actively laying females. Fluctuations in stage distribution of oocytes in the *D. pseudoobscura* ovary can be correlated with the periodicity in egg laying characteristic of this species. A mechanism to explain this egg laying rhythm is suggested.

Acknowledgments

The authors are grateful to Mrs. Ann C. Rubinson for her conscientious assistance.

References

1. Bucher, N. 1957. Experimentelle Untersuchungen über die Beziehungen zwischen Keimzellen und somatischen Zellen im Ovar von *Drosophila melanogaster*. *Rev. Suisse de Zool.*, **64**, 91-188.
2. Day, M. F. 1943. The function of the corpus allatum in muscoid Diptera. *Biol. Bull.*, **84**, 127-140.
3. Donald, H. P., & Lamy, R. 1936-1937. VI. Ovarian rhythm in *Drosophila*. *Proc. Roy. Soc. Edinburgh*, **57**, 78-96.
4. King, R. C. 1955. A method for studying successive batches of eggs laid by treated or control *Drosophila melanogaster*. *Am. Nat.*, **89**, 369-370.

5. ———, Rubinson, A. C., & Smith, R. F. 1956. Oogenesis in adult *Drosophila melanogaster*. Growth, **20**, 121-157.

6. ———, & Wood, E. M. 1955. Sex-linked lethal mutations induced by thermal neutrons in male and female *Drosophila melanogaster*. *Genetics*, **40**, 494.

7. Vogt, M. 1943. Zur Produktion gonadotropen Hormones durch Ringdrüsen der ersten Larvenstadiums bei *Drosophila*. *Biol. Zentr.*, **63**, 467-470.

8. Wigglesworth, V. B. 1936. The function of the corpus allatum in the growth and reproduction of *Rhodnius prolixus* (Hemiptera). Quart. *J. Micr. Sci.*, **79**, 91-121.

9. Thomsen, E. 1952. Functional significance of the neurosecretory brain cells and the corpus cardiacum in the female blow fly, *Calliphora erythrocephala Meig*. *J. Exp. Biol.*, **29**, 137-172.

Growth, 1957, **21**, 287-295.

ACTIVITY OF CERTAIN METABOLIC ENZYMES DURING DEVELOPMENT OF THE CHICK EMBRYO[1, 2]

JOSEPH DAVIDSON

University of Kansas,[3] Lawrence, Kansas

(Received December 5, 1957)

INTRODUCTION

STUDIES correlating biochemical, morphological and physiological events which occur during embryonic life have been undertaken in diverse species by various investigators (6, 8, 16). The work herein described is an attempt to analyze further the biochemical modifications associated with visible changes occurring throughout development of the chick embryo. The recent description (10) of a series of microchemical enzymatic assays adaptable to the minute quantities of tissue available has made it possible to carry out these biochemical determinations on separate embryonic organs.

Representative enzymes active in the glycolytic cycle, the tricarboxylic acid cycle and the electron transport system were selected. These were aldolase, succinic dehydrogenase and cytochrome oxidase respectively. Tissues selected for this study were taken from portions of the central nervous system and from the developing heart and liver. It was felt that the results of such a survey would give an indication of the basic metabolic patterns occurring throughout the development of dissimilar structures.

EXPERIMENTAL

Material and Methods

Preparation of tissues—Embryos were removed from fertile New Hampshire Red eggs incubated at 37.5° C for various time intervals (1-21 days) and the degree of development of each embryo was evaluated according to the Hamburger-Hamilton stage series for the chick (9). Samples of embryonic brain, liver and heart were removed for

[1] This work has been supported in part by U. S. Public Health grant B-439.

[2] Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at the University of Kansas.

[3] Department of Anatomy, University of Minnesota, present address.

enzyme analysis. Brain samples were divided into the 3 major divisions, hind-brain, mid-brain, and fore-brain. These tissues were suspended in water or phosphate buffer maintained at a temperature less than 5° C and then homogenized in the cold with a glass microhomogenizer. The total final volume of the homogenate was approximately 50 microliters. Since preliminary studies indicated no decrease of aldolase activity in frozen homogenates, the material to be used in the aldolase analysis was stored in a deep freeze maintained at —30° C. In contrast, cytochrome oxidase and succinic dehydrogenase activities were run on fresh tissue samples within fifteen minutes following their removal from the embryo.

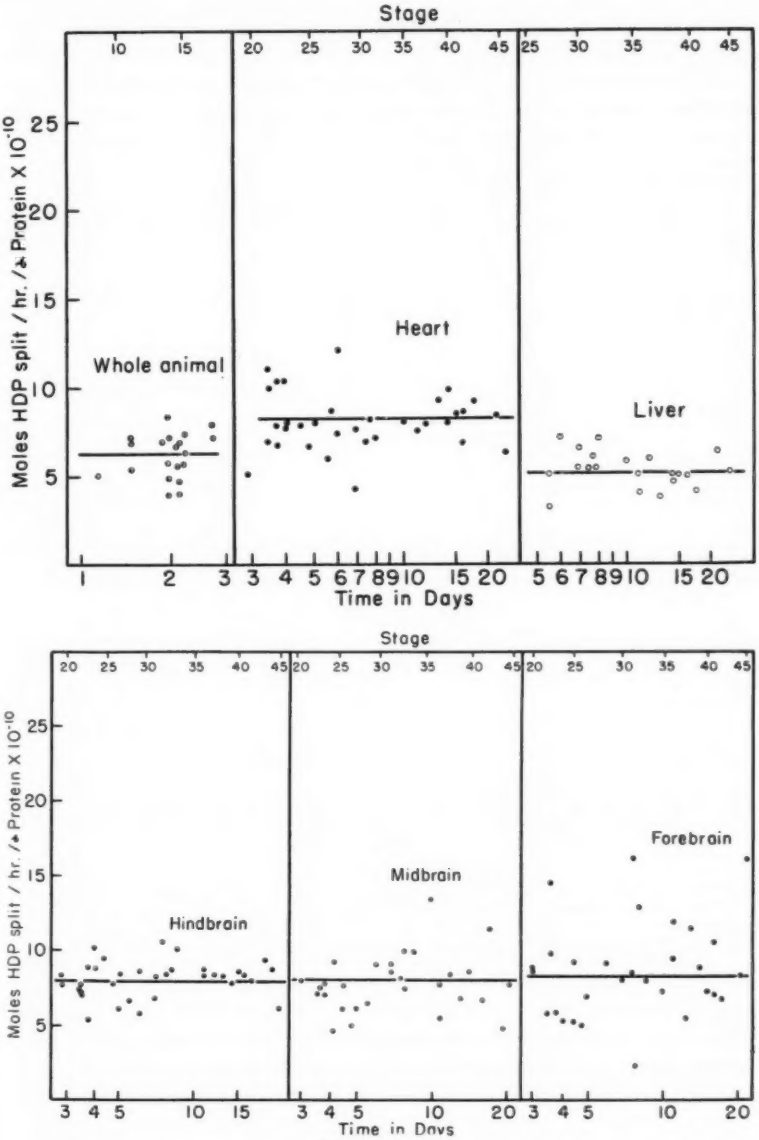
Enzyme Assay

Aldolase activity was assayed by the Lowry (10) micromodification of the dinitrophenylhydrazine method. In our procedure, approximately two to four microliters of the homogenate containing one to five micrograms of protein were incubated for the prescribed time in the substrate reagent mixture. The level of aldolase activity was expressed in units of "moles of hexosediphosphate split/hour/microgram protein." This was derived from the molar extinction data given by Lowry. Appropriate changes in these calculations were made for differences in homogenate volume.

The microspectrophotometric method of Cooperstein and Lazarow (2) was used for the quantitative determination of cytochrome oxidase. The cytochrome oxidase content of the tissues was expressed in terms of "enzyme activity/microgram protein." The standard enzyme activity was calculated according to the original paper. This notation (enzyme activity) is proportional to the slope of a line measuring the drop in a given concentration of reduced cytochrome c per unit time. A similar technique was utilized for succinic dehydrogenase determinations (3), and the apparent content of this enzyme in the tissues was likewise expressed as enzyme activity/microgram protein. For each sample a protein determination was made by use of the micromethod described by Lowry (11).

Results

The level of aldolase activity in all the organs selected for this survey remained constant during the period of development studied (Figures 1 and 2). Although the enzyme content of the individual



FIGURES 1-2

Aldolase activity in various embryonic chick organs during period of incubation studied in text. Time of development assessed by Hamburger-Hamilton (1951) stages series.

tissues appeared to be unchanging with respect to time, there were differences in activity between the organs. Heart, liver and all parts of the brain studied, varied significantly from the early whole animal. Aldolase activity of heart and brain was statistically different from that of liver, whereas brain and heart did not differ in the enzyme level.

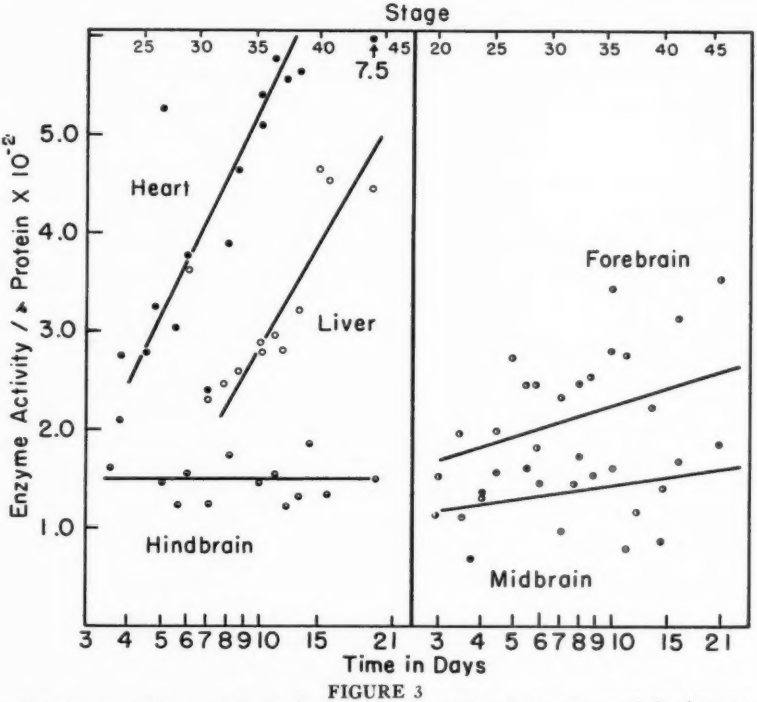


FIGURE 3

Cytochrome oxidase activity levels of embryonic organs during stages of development indicated. Time assessed by Hamburger-Hamilton (1951) stage series for chick embryo.

In contrast to the constant aldolase level in the tissues, cytochrome oxidase exhibited a marked increase in activity throughout the development of certain organs (Figure 3). The level of activity of this enzyme increased early in the embryonic heart, doubling in apparent content in the period from the fifth day to the eleventh day of incubation. This continuous rise in enzyme activity resulted in a threefold

increase by the time of hatching over the level observed at four days. Liver cytochrome oxidase activity exhibited a steady increase per unit protein throughout the developmental period. Although the rise in the liver activity lagged behind that of heart, the rate of accumula-

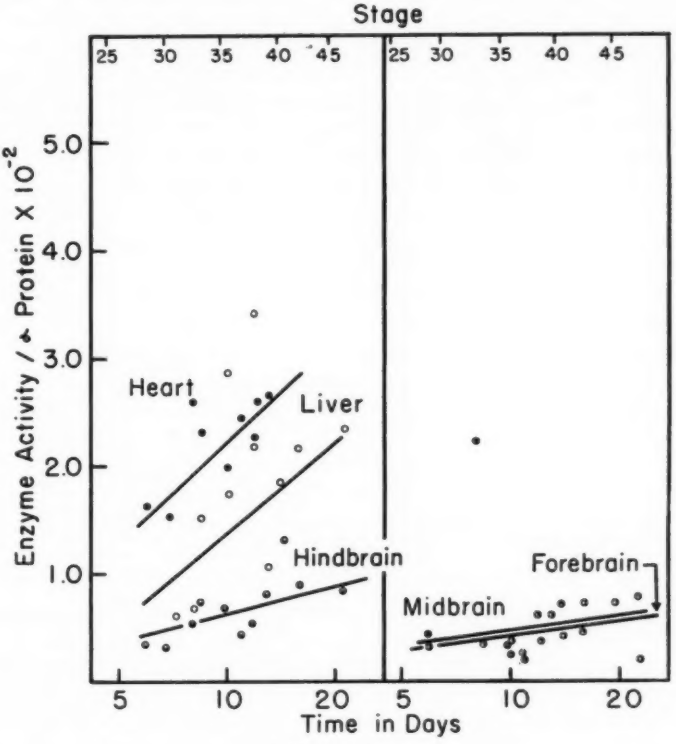


FIGURE 4

Succinic dehydrogenase activity levels of embryonic organs during stages of development indicated. Time assessed by Hamburger-Hamilton (1951) stage series for chick embryo.

tion appeared surprisingly similar in both liver and heart. In a qualitative sense, the results of the investigation of succinic dehydrogenase content were much the same as the cytochrome oxidase study (Figure 4). In fact the ratio of cytochrome oxidase activity to succinic de-

hydrogenase activity remained surprisingly constant for each individual tissue throughout the stages of development. Levels of both of these enzymes changed very slightly in brain samples taken throughout the embryonic period. A representative portion of the results of the total study is presented in Table 1.

TABLE 1
Comparative enzyme activities in chick embryonic tissues during development

Organ	Day of Development	Aldolase*	Cytochrome Oxidase**	Succinic Dehydrogenase**
Heart	Entire Period	8.37 ± 0.33		
	6		3.70	1.62
	10		5.39	2.00
	15		6.51	2.78
	20		7.50	
Liver	Entire Period	5.27 ± 0.22		
	7		2.28	0.61
	10		2.90	1.72
	13		3.22	1.86
	20		4.51	2.28
Brain (Midbrain)	Entire Period	8.08 ± 0.34		
	6		1.42	0.42
	10		1.45	0.49
	15		1.50	0.55
	20		1.59	0.62

* Expressed as Moles of Hexose-di-phosphate split/hour/microgram protein $\times 10^{-10}$.
** Expressed as Enzyme activity/microgram protein $\times 10^{-2}$.

Discussion

Most reports concerning enzyme levels during embryonic development do not deal with comparative patterns of accumulation of the various enzymes throughout ontogenesis nor with the variation in these patterns inherent within separate embryonic organs and tissues. The data reported in this study can be analyzed in either of these two ways.

Briefly, our results indicate an unchanging level of aldolase activity in the chick embryo throughout the entire period of observation concomitant with a marked increase in both cytochrome oxidase and succinic dehydrogenase enzyme activity levels. This constant level of aldolase agrees with a similar finding in the sea urchin egg (8) but differs from results obtained on foetal guinea pig cerebral cortex (6). If one assumes that the level of aldolase activity is proportional to the

total amount of glucose metabolized by way of the Embden-Meyerhof scheme for a given sample, as Lowry (10) has suggested, these results would indicate an unmodified level of activity for this major glycolytic cycle throughout development of the chick. Only related, direct studies on the kinetics of the cycle and estimations of other enzymes functioning in the scheme can reveal the validity of this assumption. Such surveys are presently being undertaken in this laboratory.

The difference in rates of accumulation of the enzymes cytochrome oxidase and succinic dehydrogenase as compared to aldolase particularly in heart and liver is suggestive. This difference indicates the possibility of an increasing amount of intermediates entering the tricarboxylic acid cycle not derived from the Embden-Meyerhof glycolytic cycle during the later stages in development. A postulate such as this is compatible with the changing respiratory quotients of developing embryos early noted by Needham (13) and explained on the basis of successive utilization of carbohydrate, protein and fat as energy sources. That interpretation is further supported by Romanoff's (15) observation that there is less dependence upon the presence of glucose as a substrate in older embryonic tissues than in young ones. A second alternative explanation is one based on a changing scheme of carbohydrate metabolism from an anaerobic one to an aerobic one. This would account for the greater participation of the enzymes involved in the oxidative degradation of pyruvate as development proceeded. Such a possibility has been suggested for embryonic liver tissue (14).

The varied pattern of enzyme accumulation observed when the separate embryonic organs are compared is of particular interest. Many chemical embryologists have offered the opinion that differences in the enzymatic make-up of developing tissues are directly related to their functional role within the adult organ and have reported on the accumulation of tissue specific enzymes to support this contention (1, 12). Our observations test the inverse situation, i.e., activities of three general metabolic enzymes in diverse tissues during embryogenesis. It is of interest to note that at the earliest dissectable stages, liver possesses lower aldolase activity than does heart or brain thereby correlating with the findings for aldolase content of these tissues in the adult (5).

The increase in cytochrome oxidase and succinic dehydrogenase activity in the heart and liver in contrast to brain indicates that these

particular enzymes do not remain at a uniform level in all tissues throughout embryonic life. However, one might anticipate that since they mediate the final stages in the release of energy, their activities would differ with the varied physiologic patterns of unlike tissues even during development. In this respect, Sippel (16) correlated the increase in the succinoxidase system of developing heart ventricle with the functional onset of cardiac activity and actual cardiac effectiveness and described parallel courses of development for the enzyme activities and the mechanical efficiency of the organ. Our observations of two components of the succinoxidase system in heart samples support this finding. Applying the same consideration of embryonic function our data on liver reveal certain similarities. The rise in activity of liver cytochrome oxidase is apparent after the seventh day of incubation. This increase occurs at the time the liver cells are considered to give the first indication of their definitive function (4). On the other hand, the presence of a relatively stable pattern for the three enzymes in all parts of the brain might well indicate the existence of a constant level of activity for this tissue throughout the greater part of development. A review of the literature failed to reveal any direct studies related to this problem in the chick. However, the work of Flexner, et al., (7) on guinea pigs is most suggestive. They found no indication of organized electrical patterns in the cerebral cortex until the third trimester of development. A similar study in the chick central nervous system would be extremely valuable in relating the metabolic pattern of development to the functional one.

Taking all of the above into consideration, it is apparent that of the three general metabolic enzymes studied only aldolase maintained an unchanging level in all developing tissues studied whereas cytochrome oxidase and succinic dehydrogenase appeared to vary with the physiologic status of the individual tissues.

Summary

Microchemical methods were employed to determine the patterns of accumulation for the enzymes, aldolase, cytochrome oxidase and succinic dehydrogenase in nervous and non-nervous organs of the developing chick embryo. Aldolase activity remained at a constant level throughout development in the embryonic heart, liver and brain. Cytochrome oxidase activity showed individual patterns of accumulation in the tissues under study. Heart and liver cytochrome oxidase ac-

tivity per unit protein increased progressively throughout the period of study whereas all portions of the central nervous system under observation remained at a fairly constant level. In general, changes in succinic dehydrogenase activity during the embryonic period appear to parallel those described for cytochrome oxidase. The activity of this enzyme likewise remained constant in the brain while heart and liver displayed a progressive rise in activity per unit protein.

Acknowledgments

I wish to express my thanks to Dr. Byron S. Wenger who suggested the problem and followed it critically throughout the actual investigation.

References

1. Boell, E. J., & Shen, S.-C. 1950. The development of cholinesterase in the central nervous system of Amblystoma Punctatum. *J. Exp. Zool.*, **113**, 583-599.
2. Cooperstein, S. J., & Lazarow, A. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. *J. Biol. Chem.*, **189**, 665-670.
3. Cooperstein, S. J., Lazarow, A., & Kurfess, N. J. 1950. The microspectrophotometric method for the determination of succinic dehydrogenase. *J. Biol. Chem.*, **186**, 129-139.
4. Dalton, A. J. 1937. The functional differentiation of hepatic cells of the chick embryo. *Anat. Rec.*, **68**, 393-409.
5. Dounce, A. L., & Beyer, G. T. 1948. A new method for the determination of the enzyme aldolase. *J. Biol. Chem.*, **173**, 159-173.
6. Flexner, J. B., Greenblatt, C. L., Cooperband, S. R., & Flexner, L. B. 1956. Biochemical and physiological differentiation during morphogenesis, XIX. *Am. J. Anat.*, **98**, 129-137.
7. Flexner, L. B., Tyler, D. B., & Gallant, L. J. 1950. Onset of electrical activity in developing cerebral cortex of fetal guinea pig. *J. Neurophysiol.*, **13**, 427-430.
8. Gustafson, T. 1954. Enzymatic aspects of embryonic differentiation. *Int. Rev. Cytol.*, **111**, 277-327.
9. Hamburger, V., & Hamilton, H. L. 1951. A series of normal stages in the development of the chick embryo. *J. Morph.*, **88**, 49-92.
10. Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixon, W. S., & Crawford, E. J. 1954a. The quantitative histochemistry of brain. II. Enzymatic methods. *J. Biol. Chem.*, **207**, 19-87.
11. ———, Rosebrough, N. J., Farr, A. L., & Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.*, **193**, 265-275.
12. Moog, F. 1952. Differentiation of enzymes in relation to the functional activities of the developing embryo. *N. Y. Acad. Sci., Annals.*, **55**, 57-66.
13. Needham, J. 1931. Chemical Embryology. Cambridge, Cambridge Press.
14. O'Connor, R. S. 1954. Observations on the nature of the relationship between cell division and carbohydrate metabolism. *J. Embryol. Exp. Morph.*, **2**, 26-37.
15. Romanoff, A. 1943. Differences in respiratory activity of isolated embryonic tissues. *J. Exp. Zool.*, **93**, 1-26.
16. Sippel, T. O. 1954. The growth of succinoxidase activity in the hearts of rat and chick embryos. *J. Exp. Zool.*, **126**, 205-221.